ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

PHYTOCHEMICAL INVESTIGATION ON THE STEM BARK OF *CROTON MACROSTACHYUS* (BISANA)

BY

ZELALEM YIBRALIGN

JULY 2007

PHYTOCHEMICAL INVESTIGATION ON THE STEM BARK OF *CROTON MACROSTACHYUS* (BISANA)

A GRADUATE PROJECT SUBMITTED TO THE OFFICE OF RESEARCH AND GRADUATE PROGRAMME OF ADDIS ABABA UNIVERSITY

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BY

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DEPARTMENT OF CHEMISTRY

SCIENCE FACULTY

JULY 2007

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ABSTRACT

PHYTOCHEMICAL INVESTIGATION ON THE STEM BARK OF *CROTON MACROSTACHYUS* (BISANA)

By: Zelalem Yibralign

Advisor: Dr. Ashebir Fiseha

*The Croton macrostachyus is used for the treatment of malaria, venereal diseases, cough, diabetes, constipation, tape worms and hepatitis. The dichloromethane extract of the stem bark of Croton macrostachyus was afforded two Terpenoids: the compound with full structure which is a triterpenoid (**CM-1**) and a compound with a partial structure which is a tetratrpenoid (**CM-2**). Structural determination was accomplished by means of spectroscopic methods (IR, UV, 1D and 2D NMR).*

1. INTRODUCTION

1.1. General

The individual chemicals from which plants made are phytochemicals. Phytochemical study of plants is of the great importance in developing drugs. [1] Drugs are strictly defined as chemical substances that are used to prevent or cure diseases in humans, animals and plants. Drugs from natural products are secondary metabolites and their derivatives. [2]

Natural products have been a major source of drugs for centuries, with more than 25% of the pharmaceuticals in use today derived from natural products. [3] The natural products as medicinal agents presumably predates the earliest recorded history as the earliest humans used various, but specific plants to treat illness. Natural products are those chemical compounds derived from living organisms, plants, animals and insects. Until the late 1800's, organic chemistry was almost exclusively the study and use of natural products. [4]

Natural product chemistry is a part of organic chemistry that covers the chemistry of naturally occurring organic compounds: their biosynthesis, function in their environment, metabolism and more conventional branches of chemistry such as structural elucidation and synthesis. Primary metabolism is the system of biochemical reactions whose products are vital for the living organisms. Primary metabolic path ways often function in cycles. Secondary metabolism refers to the functions of an organism yielding products that are not necessary for the essential biochemical events. Secondary metabolites are thus compounds which are often species dependent.[5, 6] The secondary metabolites of organisms, including plants, serve important biological and ecological roles, mainly as chemical messengers and for defense purposes. [7] Primary metabolites are essentially ubiquitous and certainly essential for life, whilst the secondary metabolites are of restricted occurrence and of no apparent utility. [8] In principle, the secondary metabolites are non essential to life but they definitely contribute to the species' fitness of survival. [9] The classification of natural products, which cover almost all types of organic molecules, can be based on: chemical structure, physiological activity, taxonomy, and biogenesis. [10]

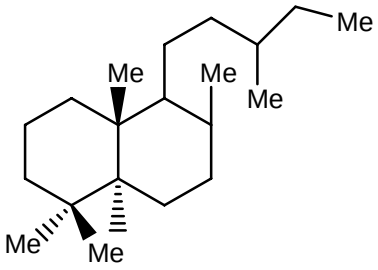
The World Health Organization (WHO) estimates that around 80% of the world population in developing countries relies on traditional medicines for primary health care needs, of which a major proportion corresponds to plant extracts or their active principles. [11]

1.2. Croton species and their medicinal uses

The Euphorbiaceae are mostly monoecious herbs, shrubs, and trees, sometimes succulent and cactus-like, comprising one of the largest families of plants with about 300 genera and 7,500 species that are further characterized by frequent occurrence of milk sap. [12] The Species of Euphorbiaceae family are grown mainly in tropical regions. The major genera of Euphorbiaceae family are Euphorbia, *Croton*, Phyllanthus, Acalypha, Glochidion, Macaranga, Drypetes and Jatropha. [13]

The genus *croton* which belongs to the family Euphorbiacea and sub family crotonoideae has about 1,300 species of trees, shrubs and herbs distributed in tropical and subtropical regions of both hemispheres. In the state of Rio de Janeiro alone 39 species have been identified. The genus *croton* is particularly rich in secondary metabolites like alkaloids, terpenoids, and flavonoids.[14] The most common class of compounds of croton is represented by diterpenoids. Apparently, clerodane is the widest spread class of diterpenoids in croton, which has been found in species from America (e. g. *C. cajucara*), Africa (e. g. *C. macrostachyus*) and Asia (e. g. *C. tiglium*). The genus is also rich in constituents with biological activities, chiefly diterpenoids such as labdane (**1**) clerodane (**2**), kaurane (**3**), trachylobane (**4**) and pimarane (**5**). Several species of the genus are aromatic, indicating the presence of volatile oil constituents. As most Euphorbiaceae, croton species may contain latex, which is red-coloured in some species, a characteristic usually with medicinal properties. Several croton species have a long role in the traditional use of medicinal plants in Africa, Asia and South America. Popular uses include treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, leukemia, balsamic, narcotic, rheumatism, stomachic and tonic, bronchitis, diarrhea, leprosy, psoriasis, urtcaria, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain ulcers and weight loss. Some of the croton

species which are used for the treatment of such diseases traditionally in different worlds include: *C. cajucara* Benth., popularly known as "sacaca", *C. celtidifolius* Baill., commonly known as "sangue-de-adave", *C. eluteria* Benett., commonly known as "cascarilla", *C. malambo* Karst., *C. nepetaefolius* Baill., *C. palanostigma* Klotzsch, *C. schiedeanus* Schlecht., *C. uraucurana* Baill. , *C. zehntneri* Pax. Et Hoffm. (in South America), *C. arboreous* Millsp., *C. californicus* Mull. Arg., *C. draco* Cham. & Schldl. (in North America and Central America), *C. macrostachyus* Hochst. ex Rich., *C. zambesicus* Mull. Arg (synonyms to *C. amabilis* Mull. Arg.; *C. gratissimus* Burch.) (in Africa), *C. kongensis* Gagnep., *C. oblingifolius* Roxb., popularly known as "chukka", *C. sublyratus* Kurz., *C. tiglium* L., and *C. tonkinensis* Gagnep, popularly called "Kho sam Bac Bo" (in Asia). The parts of those croton species which are used for medicinal purpose for treatment of different kinds of diseases are the leaves, the roots, the stem barks and the fruits. [11]



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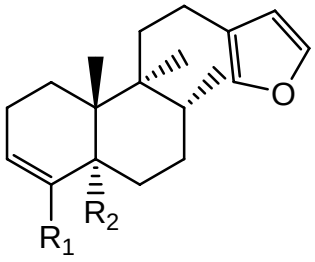

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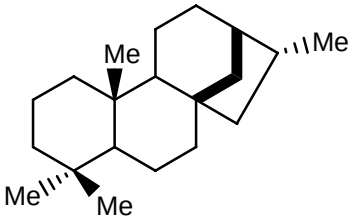

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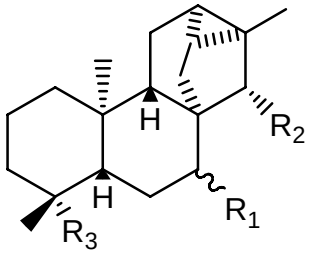

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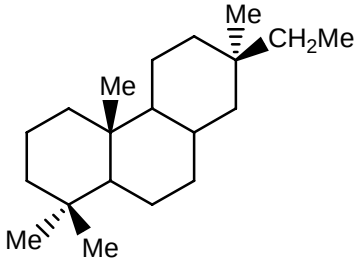


5

2. TERPENES

The terpenes are ubiquitous metabolites found in all living organisms. They include essential metabolites such as the sterols acting as membrane stabilizers in eukaryotes or precursors for steroid hormones. [15] They are among the most wide spread and chemically diverse groups of natural products throughout the plant and animal kingdom. [1, 16] They form a large and structurally diverse family of the natural products derived from C_5 isoprene units joined in a head-to-tail fashion. [17] These diverse, wide spread, and exceedingly numerous family of natural products are synonymously termed as terpenoids, terpenes, or isoprenoids. They are typically found in all parts (i. e., seed, flowers, foliage, roots, wood) of higher plants and also occur in mosses, liver worts, algae, and lichens, although some are of insect or microbial origin. [8] Terpenes containing 30 carbons or more are usually formed by the fusion of two smaller terpene precursors such that the head-to-tail "rule" appears to be violated. [18] They are found in abundance in higher plants and many terpenes occur as glycosyl esters, iridoid and triterpene glycosides being the most abundant. [19]

2.1. Classification of Terpenes

Despite their structural diversity, terpenes have a simple unifying feature by which they are defined and by which they may be easily classified. They are group of hydrocarbon-based natural products whose structure may be derived from isoprene, giving rise to structures which may be divided into isopentane (2-methyl butane) units. Terpenes thus can be classified by the number of 5-carbon units they contain. [1] These include:

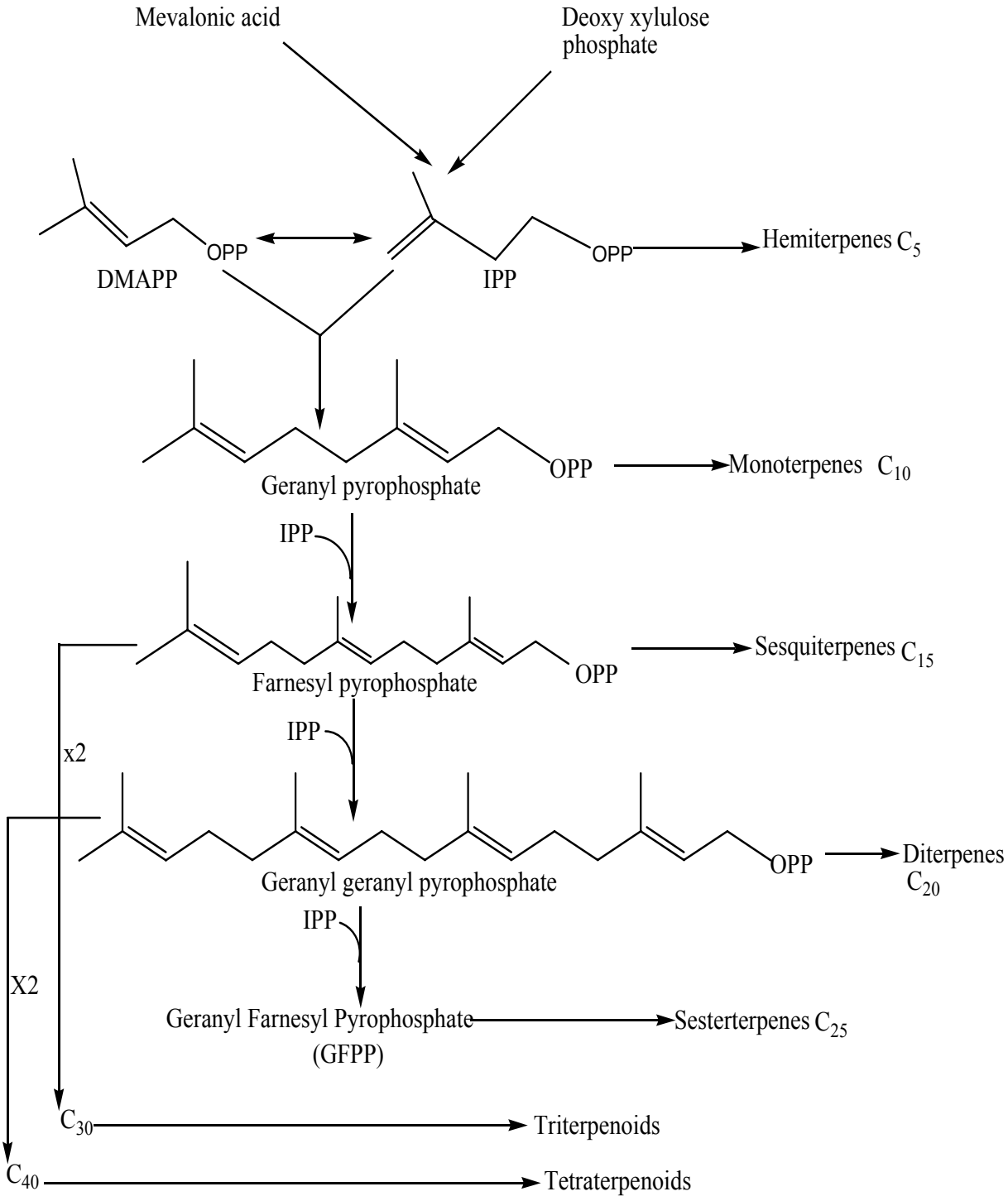
- Hemiterpenes (C_5)
- Monoterpenes (C_{10})
- Sesquiterpenes (C_{15})
- Diterpenes (C_{20})
- Sesterterpenes (C_{25})
- Triterpenes (C_{30})
- Tetraterpenes (C_{40})

Volatile monoterpenes contribute to flavours and aromas in food and are important in perfumery. The importance of monoterpenes in flavouring has renewed interest in their biosynthesis and bioformation. The monoterpenes and their glycosides in grapes contribute to the aromas of wine. Many flavour components arise by modification of terpenes either biosynthetically or during chemical flavour generation process. [19] Monoterpenes include acyclic, monocyclic, bicyclic and tricyclic types. A large percentage of them occur in higher plants as hydrocarbons, but alcohols, aldehydes, ketones, acid lactones, oxides and peroxides have been found. Most of them can be divided into head-to-tail combination of two isopentane units. [1]

2.2. Biosynthesis of Terpenes

The reaction path leading to a particular natural product is called the biosynthetic path way, and the corresponding event is known as the biogenesis. Different plant and animal species can employ dramatically different biosynthetic path ways to produce the same metabolite. This feature can be employed in the classification of plants in terms of their chemotaxonomy. [6]

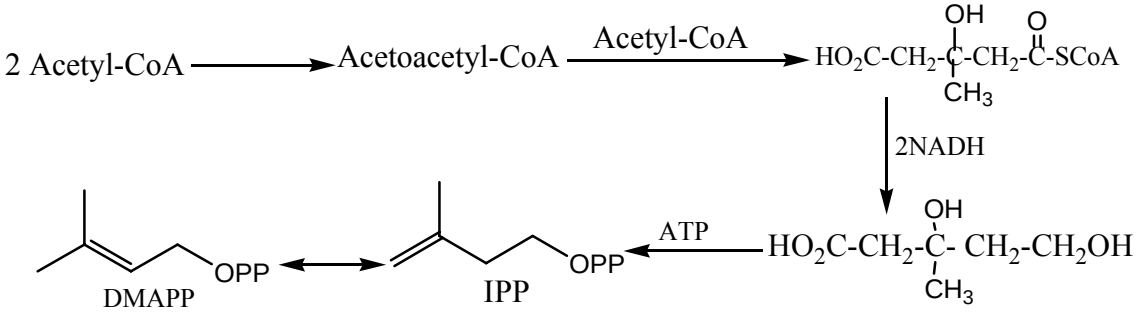
Isoprenes had been characterized as a decomposition product from various natural cyclic hydrocarbons, and were suggested as the fundamental building block of these compounds, also referred to as 'isoprenoids'. Isoprene is produced naturally but is not involved in the formation of these compounds, and the biochemically active isoprene units were identified as the diphosphate (pyrophosphate) esters, dimethyl allyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). The biochemical isoprene units may be derived by way of intermediates mevalonic acid (MVA) or 1-deoxy-D-xylulose-5-phosphate (deoxyxylulose phosphate=DXP). [17]



Scheme 1: Biosynthesis of Terpenes

2.2.1. Biogenesis of Isopentenyl pyrophosphate (IPP)

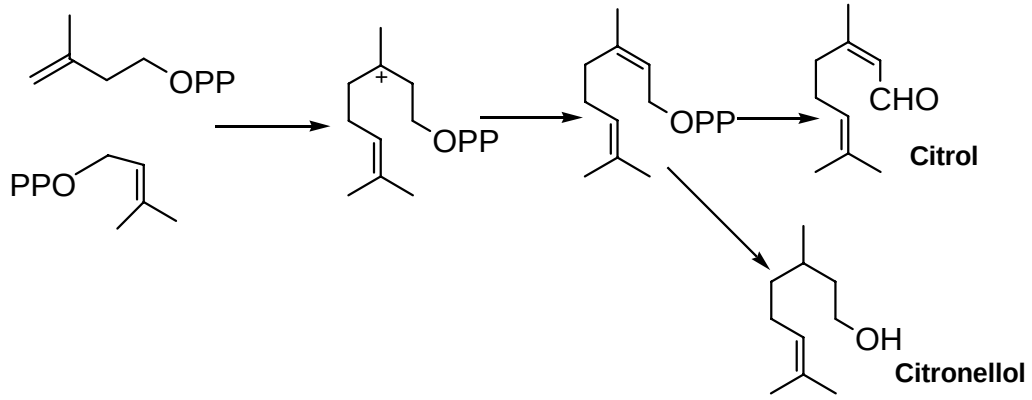
Isopentenyl diphosphate (IPP), the common precursor of all isprenoids, was generally believed to arise from acetyl coenzyme A, 3-hydroxy-3-methyl glutaryl-coenzyme A and mevalonate. [20] It is formed by two different biosynthetic routes, the well-known acetate (mevalonate) path way that was long and unanimously accepted as the sole biosynthetic route to IPP, and the mevalonate-independent path way starting from pyruvate and glyceraldehyde-3-phosphate yielding IPP.[21, 22] Isopentenyl and DMAPP are those reactive species which are formed from mevalonic acid by phosphorylation by ATP-assisted loss of water and carbon dioxide. [6]



Scheme 2: Biogenesis of IPP (the Mevalonate path way)

2.2.2. Biogenesis of Monoterpenes

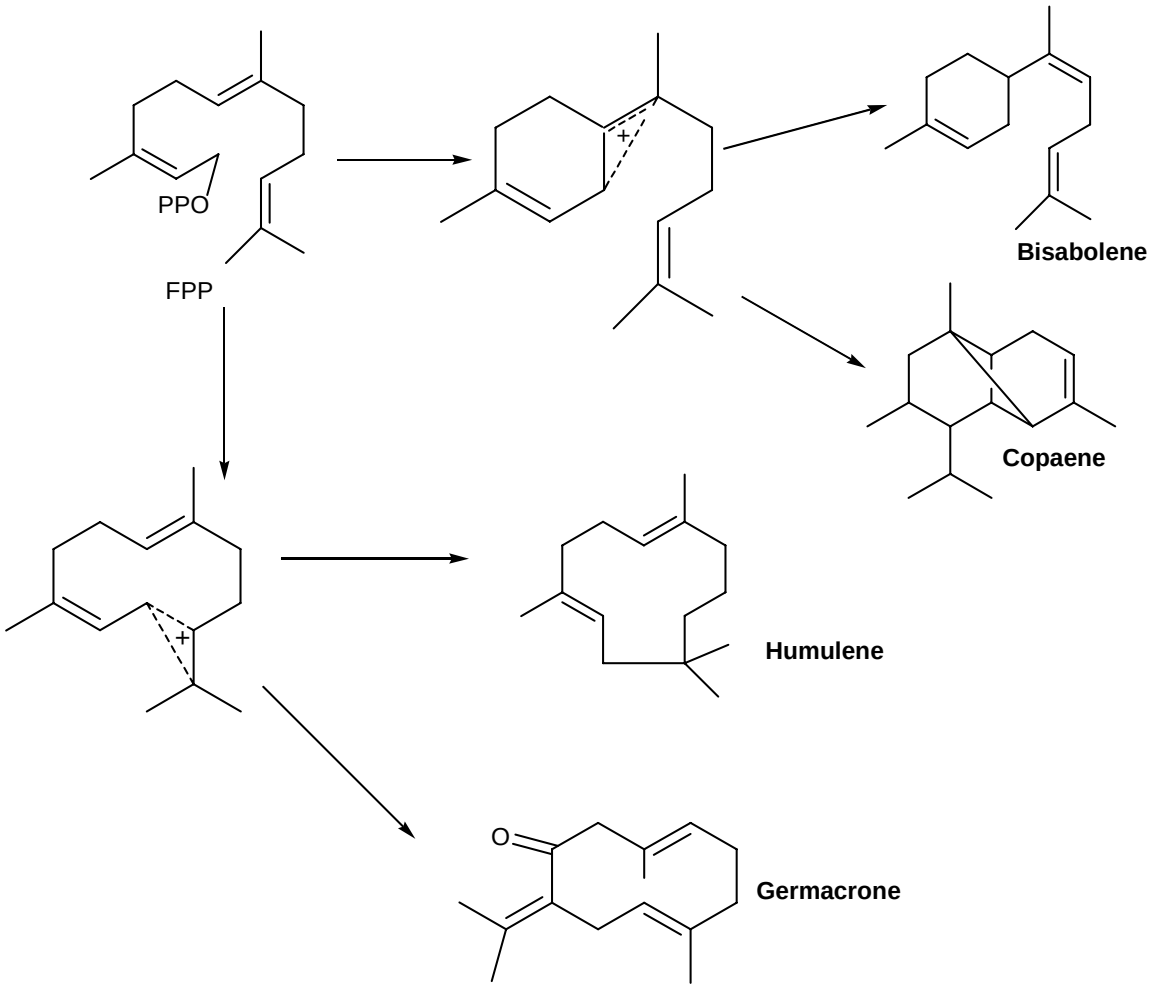
Monoterpesens are acyclic or cyclic C_{10} hydrocarbons and their oxygenated derivatives. They are widely used in the flavour and perfume industries because of their attractive odours, low molecular weights and high volatilities. [23]



Scheme 3: Biogenesis of Monoterpenes

2.2.3. Biogenesis of Sesquiterpenes

Sesquiterpenes are derived from three isoprene units. They are C_{15} hydrocarbons or their oxygenated analogues. They exist in a wide variety of forms, including linear, bicyclic, and tricyclic frame works. Like the monoterpenes, most of them are considered to be essential oils because they belong to the stem distillable fraction often containing the characteristic odoriferous components of the plant. [1] Sesquiterpenes are formed from cis-trans farnesyl pyrophosphate (FPP) through cationic cyclization reactions and subsequent rearrangement of the resulting carbonium ions. [6, 24]



Scheme 4: Biogenesis of Sesquiterpenes

3. CROTON MACROSTACHYUS

3.1. Botanical Back ground

Croton macrostachyus which belongs to the family Euphobiaceae is a medium sized deciduous tree of East Africa particularly wide spread between 200-2500 m in mountainous forests and savannah of the tropical regions and ever green bush land areas that receive between 700-2000 mm rainfalls annually. [25, 26] The plant is also identified by the synonyms *Croton accuminatum* R. Br. and *Rottlera schimperi* Hochst. Ex. A. Rich. [27] The generic name of the plant is derived from the appearance of the seed, for '*Croton*' is based on the Greek word for a tick. The specific epithet is from the Greek macro- (large) and –stachyus (relating to spike) hence "with a large spike". These trees experience extended flowering seasons in most areas, peaking in March-June and May-July, providing excellent bee forage. For instance, in Kenya, flowering is observed in Kakamega District in March and April; in Nyeri, Meru and Kericho Districts in June and July; and in Pokot District in August and September. In Nigeria, flowering occurs in March to May and fruiting from January to March. After pollination by insects, fruit development takes place 3-5 months. *Croton macrostachyus* is 3-25 m high, although more commonly 6-12 m. It is common in secondary forests, on forest edges along rivers, wood lands, wooded grass lands or clump bush land and along road sides. It is associated with Janiperus Podocarpus habitats and also occurs in the warmer parts of the montane rain forests and semi-tropical rain forests. Outside the forests, in wetter areas, the species is widely distributed. It is native to Eritrea, Ethiopia, Kenya, Tanzania, Uganda and Nigeria. *Croton macrostachyus* is employed in soil conservation, trees are commonly planted for the useful shade that they provide, leaf fall provides mulch and green manure; the cream-coloured, soft wood is used for indoor carpentry, furniture, veneers, tool handles, boxes and crates. [28]

Many parts of the *croton macrostachyus* have medicinal value including boiled leaf decoction is drunk or ashes taken orally as treatment for cough; juice from fresh leaves is applied on wounds to hasten clotting. Roots are used as an anthlemintic for tape worm, for malaria, venereal diseases, as antidiabetic, and the seeds are widely used as purgative, for constipation and for stomach worms. Bark from stems and roots is boiled in water and

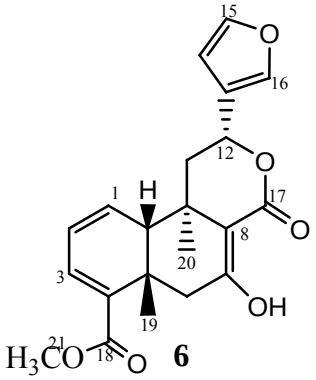
newly born babies are bathed in the mixture as a remedy for skin rash. The leaves of the tree are also used for fodder and the tree is used for shade. [29] The stem bark and the tips of the different branches of the tree are used for the treatment of malaria and hepatitis in different parts of Ethiopia, particularly in Gojjam in the Amhara Regional State.

3.2. Secondary Metabolites from *Croton macrostachyus*

Phytochemical study on the genus *Croton* has lead to the isolation and characterization of different classes of secondary metabolites. Terpenes, flavonoids and alkaloids have been isolated from the different *croton* species. Terpenoids are the predominant secondary metabolite constituents in the genus, chiefly diterpenoids, which may belong to the clerodane, neoclerodane, kaurane, labdane, phorbol and trachylobane skeletal types. Triterpenoids, either pentacyclic or steroidal, have frequently been reported for *croton* species.

Table 1: Some Terpenes isolated from *Croton macrostachyus*

NO.	Name of the compound	Structures	The source	Reference
1.	Crotomacrine	**6**	Fruits	26
2.	Crotepoxide	**7**	Fruits	26
3.	3β-acetoxy tetraxer-14-en-28-oic acid	**8**	Roots	25
4.	Trachyloban-19-oic acid	**9**	Roots	25
5.	Trachyloban-18-oic acid	**10**	Roots	25
6.	Neoclerodan-5,10-en-19,6β; 20, 12-diolide	**11**	Roots	25
7.	3α,19-dihydroxy trachylobane	**12**	Roots	25
8.	3α,18,19-trihydroxy trachylobane	**13**	Roots	25
9.	Lupeol	**14**	Root Bark	27



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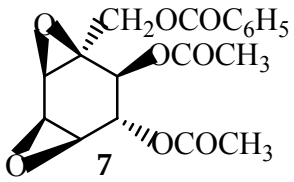
Crotomacrine



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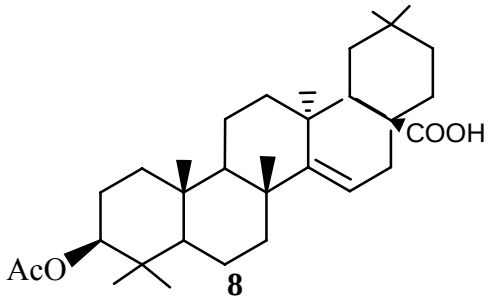
Crotepoxide



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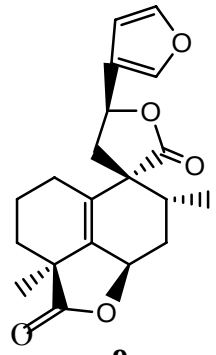
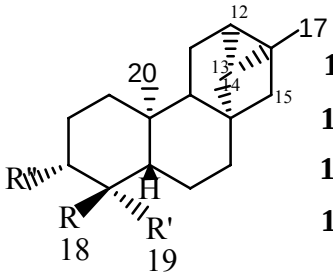
3β-acetoxy tetraxer-14-en-28- oic acid



9

Trachyloban-19-oic acid

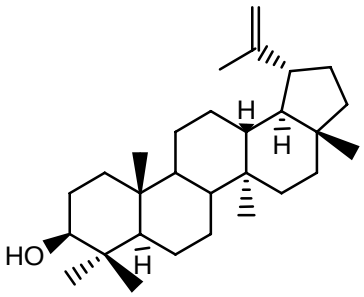


10. R= CH_3, R'= COOH, R"= H

11. R= COOH, R'= CH_3, R"=H

12. R=CH_3, R'= CH_2OH, R'=OH

13. R= CH_2OH, R'= CH_2OH, R"=OH



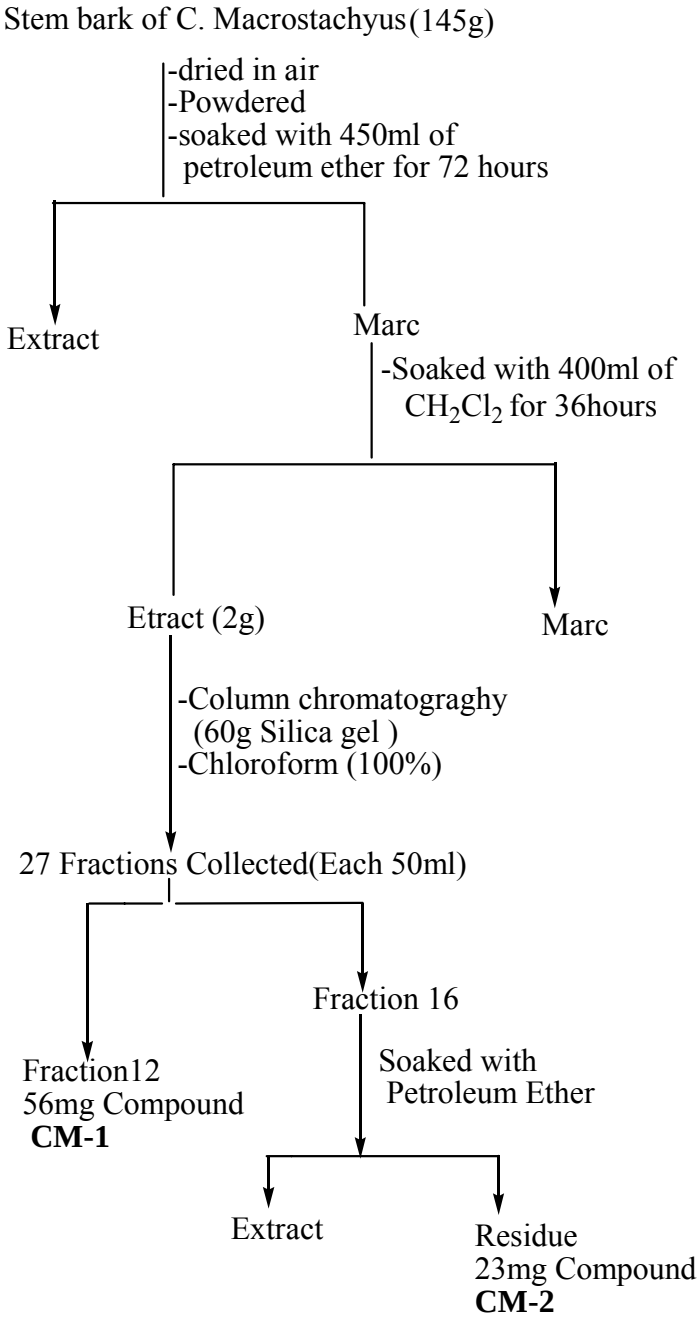
14 (Lupeol)

4. OBJECTIVES OF THE STUDY

The main objective of this project was isolation and structural elucidation of the constituents of the stem bark of *Croton macrostachyus*. The plant was selected for this study because it is important in traditional medicine for the treatment of malaria, hepatitis, and different kinds of diseases.

5. RESULTS AND DISCUSSION

The air dried and powdered stem bark of the *Croton macrostachyus* was extracted with dichloromethane. This extract when developed on TLC and sprayed with 1% vanillin sulphuric acid and after heating for a few minutes has shown the characteristic color change that indicated the presence of terpenes. The pale yellow organic extract was subjected to column chromatography on silica gel and 27 fractions were collected. This study has resulted in the isolation and characterization of two terpenes.The scheme of extraction is shown below:



Scheme 5: Method used to extract plant material

5.1. Characterization of compound CM-1

The compound **CM-1** was obtained as a white solid that showed a characteristic colour change to violet on TLC plate upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. This compound has RF value 0.71 using chloroform: ethyl acetate (4:1) as a solvent.

In the IR spectrum of the compound **CM-1** (Appendix 1.1), the absorption band at 3308cm^{-1} showed the O-H stretching that indicated the presence of a hydroxyl group. The absorption band at 3066 cm^{-1} showed the presence of the C-H stretching of the olefin. The strong absorption band at 2946 cm^{-1} showed the presence of the C-H stretching for methyl groups. The absorption band at 2873 cm^{-1} showed the presence of C-H stretching of the methylene groups. The absorption band at 1638 cm^{-1} showed the presence of the olefinic C=C stretching. The absorption band at 1043 cm^{-1} showed the presence of the C-O bond stretching. The UV-Vis spectrum at λ_{max} ($CHCl_3$) (Appendix 1.2), showed the absorption band at 268 nm indicated that the molecule has no conjugation.

The ^{1}H NMR spectrum (Appendix 1.3) showed two broad singlet peaks at δ 4.60 and δ 4.70, each integrating for one proton, corresponded to the methine protons of an olefinic methylene group. A doublet of doublet peak at δ 3.2 integrating for one proton corresponded to the methine proton which was assigned to C-3 carbon atom. A multiplet peak at δ 2.4 integrating for one proton corresponded to the methine proton which was attached to C-19 carbon atom. A singlet peak at δ 2.15 integrating for one proton corresponded to the –OH proton. The broad singlet at δ 1.75 integrating for three protons corresponded to C-30 at δ 19.08. The ^{1}H NMR experiment also exhibited signals due to 6 quaternary methyl protons (all singlet), with their peaks at δ 1.03, δ 0.84, δ 0.97, δ 0.95, δ 0.76, and δ 0.79, each integrating for three protons, corresponded to the carbons at δ 15.37, δ 15.98, δ 14.56, δ 27.99, δ 16.12 and δ 18.01 respectively. There are also different peaks from δ 0.7 to δ 2.0 which consist of CH- and CH_2-, totally about 25H.

The ^{13}C NMR spectrum (Appendix 1.4) of compound **CM-1** showed well resolved resonance of the 31 carbon atoms. The DEPT spectrum (Appendix 1.5) of the compound **CM-1** indicated the presence of 7 methyl, 12 methylene, 6 methine and 6 quaternary carbons.

Table 2: Proton Decoupled ^{13}C NMR and DEPT (400 MHz, $CDCl_3$) spectral data of Compound **CM-1**

Carbon No.	^{13}C NMR δ (in ppm)	DEPT δ (in ppm)	Remark
1.	38.72	38.72	CH_2
2.	27.42	27.42	CH_2
3.	79.00	79.00	CH
4.	38.87	-	C (Quaternary carbon)
5.	55.31	55.31	CH
6.	18.33	18.33	CH_2
7.	34.29	34.29	CH_2
8.	40.84	-	C (Quaternary carbon)
9.	50.45	50.45	CH
10.	37.18	-	C (Quaternary carbon)
11.	20.94	20.94	CH_2
12.	25.15	25.15	CH_2
13.	38.07	38.07	CH
14.	43.01	-	C (Quaternary carbon)
15.	27.46	27.46	CH_2
16.	35.59	35.59	CH_2
17.	42.84	42.84	C (Quaternary carbon)
18.	42.32	42.32	CH
19.	48.00	48.00	CH
20.	150.97	-	C (Quaternary carbon)
21.	29.86	29.86	CH_2
22.	40.01	40.01	CH_2

Table 2: *(Continued)*

23.	27.99	28.00	CH_3
24.	16.12	16.12	CH_3
25.	15.98	15.98	CH_3
26.	15.37	15.37	CH_3
27.	14.56	14.56	CH_3
28.	18.01	18.01	CH_3
29.	109.33	109.33	CH_2
30.	19.31	19.31	CH_3
31.	29.71	29.71	CH_2

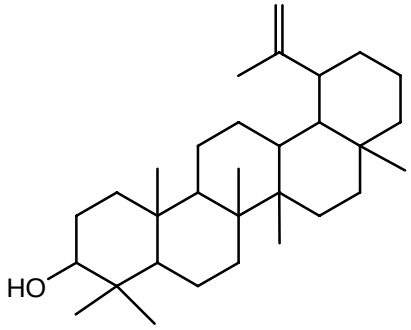
From comparison of the ^{13}C NMR spectral data of **CM-1** with that of lupeol from the literature, compound **CM-1** closely resembles lupeol. However, lupeol has 30 carbons while compound **CM-1** has 31 carbons. There is an exact match with ^{13}C NMR values of all 30 carbons as shown in the **table 3** below. Lupeol has about 11 methylene groups but the compound **CM-1** has about 12 methylene groups. Therefore, to propose the structure for compound **CM-1**, slight modification was needed to get the 12^{th} methylene group based on the structure from the literature. The 12^{th} methylene group was added between C-21 and C-22 from lupeol. An evidence for the addition of the 12^{th} CH_2 group between the two carbons was obtained from 2D spectral data.

Table 3: Proton Decoupled ^{13}C NMR (400 MHz, $CDCl_3$) spectral data of Compound **CM-1** (base skeleton) compared with literature. [30, 31]

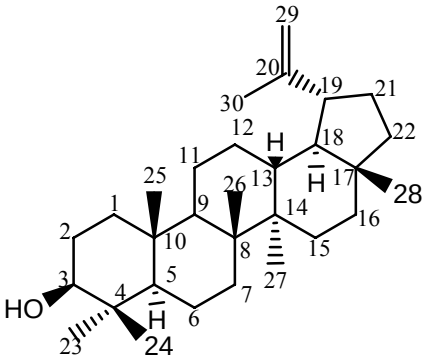
Carbon No.	**CM-1**	**Lupeol**
1.	38.72	38.70
2.	27.42	27.36
3.	79.00	78.65
4.	38.87	38.85
5.	55.31	55.26
6.	18.33	18.29

Table 3: *(Continued)*

7.	34.29	34.26
8.	40.84	40.80
9.	50.45	50.34
10.	37.18	37.11
11.	20.94	20.97
12.	25.15	26.53
13.	38.07	37.96
14.	43.01	42.97
15.	27.46	27.36
16.	35.59	35.44
17.	42.84	42.74
18.	48.32	48.72
19.	48.00	43.77
20.	150.97	151.32
21.	29.86	31.70
22.	40.01	39.83
23.	27.99	28.03
24.	16.12	16.11
25.	15.98	15.96
26.	15.37	15.48
27.	14.56	14.52
28.	18.01	17.69
29.	109.33	109.67
30.	19.31	19.65
31.	29.71	-



CM-1



Lupeol

In addition to the above informations which were used to compute the chemical shifts of **CM-1** with the literature value, there are also 2D experiments which were used to support the above predictions for given structure of compound **CM-1**.

The ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum (Appendix 1.6) showed the strong correlations between H-29 at δ 4.60 and δ 4.70 and H-30, indicating that the two methylene protons at the terminal olefinic carbon are diastereotopic. Similarly, there are also other strong couplings which were observed from COSY experiment as shown below in **table 4**.

Table 4: ^{1}H NMR and some selected ^{1}H-^{1}H COSY (400 MHz, $CDCl_3$) spectral data of compound **CM-1**

Carbon No.	^{1}H NMR δ (in ppm)	^{1}H-^{1}H COSY
1.	0.90 and 1.6	
2.	1.61	$H^2 \leftrightarrow H^3$
3.	3.20	$H^3 \leftrightarrow H^2$
5.	0.70	$H^5 \leftrightarrow H^6$
6.	1.68	$H^6 \leftrightarrow H^5$
7.	1.39	
9.	1.30	

Table 4: *(Continued)*

11.	1.46	$H^{11} \leftrightarrow H^{12}$
12.	1.64	$H^{12} \leftrightarrow H^{11}$
13.	1.61	
15.	1.60	$H^{15} \leftrightarrow H^{16}$
16.	1.52	$H^{16} \leftrightarrow H^{15}$
18.	1.40	$H^{18} \leftrightarrow H^{19}$
19.	2.40	$H^{19} \leftrightarrow H^{21}$
21.	1.90 and 1.24	$H^{21} \leftrightarrow H^{19}$ and H^{31}
22.	1.20	
23.	0.95	
24.	0.76	
25	0.84	
26.	1.03	$H^{26} \leftrightarrow H^{7}$
27.	0.97	$H^{27} \leftrightarrow H^{15}$
28.	0.79	
29.	4.6 and 4.7	H^{29} (4.6) $\leftrightarrow H^{30}$and H^{29}(4.7) $\leftrightarrow H^{19}$
30.	1.75	$H^{30} \leftrightarrow H^{29}$
31.	1.24	

Heteronuclar Single Quantum Correlation (HSQC) spectrum (Appendix 1.7) also correlates the chemical shift of proton with directly bonded carbon atom. The HSQC NMR spectrum showed the important correlations such that, the two diastereotopic methylene protons at δ 4.6 and δ 4.7 correlate with that of carbon at δ109.33. This correlation showed that this carbon is terminal olefinic carbon. Other diastereotopic methylene protons at δ 1.24 and δ 1.90 correlate with that of carbon at δ 29.86. The diastereotopic methylene protons at δ 1.60 and δ 0.90 also correlate with that of carbon at δ 38.72. In the same manner, the HSQC spectrum also showed different kinds of correlations of the protons with that of carbons as shown in Appendix 1.7.

Another experiment which was used to propose the structure of **CM-1** in addition to the above experiments was HMBC (Appendix 1.8). From this HMBC spectrum there are correlations of protons with carbons which are two or three bonds away. Some of the correlations which were observed from the HMBC experiment can be shown in the following table below:

Table 5: Observed correlations in HMBC (400 MHz, $CDCl_3$) spectral data of compound **CM-1**

Carbon No.	^{13}C NMR δ (ppm)	HMBC
1.	38.72	$H^1 \leftrightarrow$C-25
2.	27.42	$H^2 \leftrightarrow$ C-3
3.	79.00	$H^3 \leftrightarrow$ C-1, C-23
4.	38.87	
5.	55.31	$H^5 \leftrightarrow$ C-24, C-25
6.	18.33	
7.	34.29	
8.	40.84	
9.	50.45	
10.	37.18	
11.	20.94	
12.	25.15	
13.	38.07	
14.	43.07	
15.	27.46	
16.	35.59	
17.	42.84	
18.	48.32	
19.	48.00	$H^{19} \leftrightarrow$C-29, C-18, C-21, C-30
20.	150.97	
21.	29.86	

Table 5: *(Continued)*

22.	40.01	
23.	27.99	$H^{23}\leftrightarrow$ C-5, C-3, C-2, C-4
24.	16.12	$H^{24}\leftrightarrow$ C-3, C-5, C-4, C-23
25.	15.98	$H^{25}\leftrightarrow$ C-5, C-9, C-10, C-4, C-1
26.	15.37	$H^{26}\leftrightarrow$ C-7, C-9, C-14, C-8
27.	14.56	$H^{27}\leftrightarrow$C-13, C-26
28.	18.01	$H^{28}\leftrightarrow$ C-18, C-17, C-22, C-16
29.	109.33	$H^{29}\leftrightarrow$ C-19, C-30
30.	19.31	$H^{30}\leftrightarrow$C-20, C-18
31	29.71	

Based on the HMBC experiment the correlations of the protons with carbons which are two or three bonds away can be shown structurally as follows:

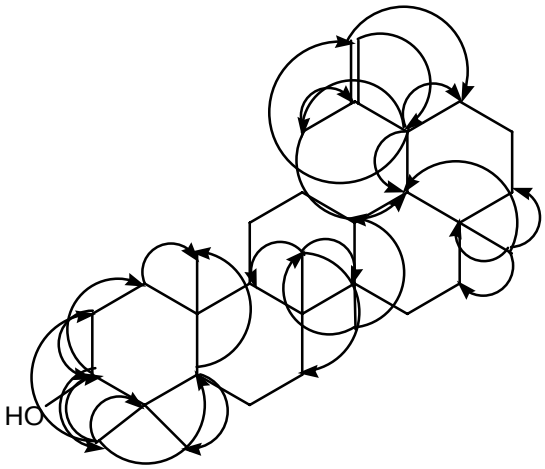
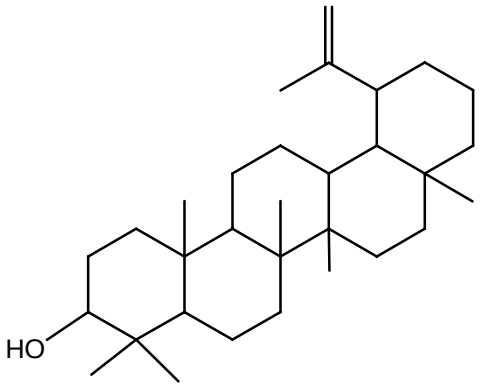


Figure 1: Observed HMBC correlations of compound **CM-1**

Based on spectroscopic data and literature, the compound **CM-1** is a triterpene with the following structure.



CM-1

5.2. Characterization of partial structure of Compound CM-2

The compound CM-2 was obtained as a white solid that showed the characteristic coloured change to violet on TLC plate upon spraying 1% vanillin sulfuric acid and after heating for a few minutes. This compound has RF value 0.68 using chloroform: ethyl acetate (7:3) as a solvent.

In the IR spectrum of the compound **CM-2** (Appendix 2.1), the absorption band at 3377 cm^{-1} showed the stretching of O-H that indicated the presence of the hydroxyl group. The absorption band at 2940 cm^{-1} showed the C-H stretching of the methyl group. The absorption band at 2868 cm^{-1} showed the C-H stretching of the methylene group. The absorption band at 1043 cm^{-1} showed the C-O stretching. The UV-Vis spectrum at λ_{max} ($CHCl_3$) (Appendix 2.2), showed the absorption band at 271 nm indicated that the molecule has conjugation with double bonds.

The ^1H NMR spectrum (Appendix 2.3) showed two broad singlet peaks at δ 4.6 and δ 4.7, each integrating for one proton, corresponded to the methine protons of an olefinic methylene which were attached to the terminal olefinic carbon at δ 109.71. These protons are called diasterotopic protons. A doublet of doublet peak at δ 3.2, integrating for one proton, corresponded to the methine proton which was attached to C-3 (δ 78.98). A multiplet peak δ 2.4, integrating for one proton, corresponded to the methine proton which was attached to C-19 (δ 48.07). A doublet peak at δ 0.70, integrating for one

proton, corresponded to the methine proton which was attached to C-5 (δ 55.28). A broad singlet peak at δ 1.78, integrating for three protons, corresponded to the C-30 which is vinylic carbon at δ 19.08. The ^{1}H NMR spectrum also exhibited signals due to 5-quaternary methyl protons with their peaks at δ 1.00, integrating for 6 protons, corresponded to the methyl protons attached with C-23 (δ 27.99) and C-27 (δ 14.76) and δ 0.78, δ 0.85, δ 1.05, each integrating for three protons, corresponded to carbon atoms at δ 16.12, δ 15.98, and δ 15.37 respectively. There are also different peaks from δ 0.90 to δ 2.00 which consist of different CH- and CH_2-groups. These interpretations showed only the partial structure of the compound **CM-2**.

Below there is a table (**table 6**) which showed the ^{13}C NMR and DEPT spectral data of the compound **CM-2**. The ^{13}C NMR spectrum of the compound **CM-2** (Appendix 2.4) showed well resolved resonance of the 40 carbon atoms. The DEPT spectrum (Appendix 2.5) indicated the presence of 13 methylene, 6 quaternary and 21 methine or methyl carbons. These figures were from the total 40 carbons of the compound **CM-2**, but from those carbon atoms which were reported in the partial structure of the compound **CM-2** the DEPT spectrum indicated the presence of 10 methylene, 6 methyl, 6 methine and 5 quaternary carbons. The carbons from a-m are those which do not assigned to the partial structure of compound **CM-2** and they are not numbered.

Table 6: Proton Decoupled ^{13}C NMR and DEPT (400 MHz, $CDCl_3$) Spectral Data of Compound **CM-2.**

Carbon No.	^{13}C NMR δ (in ppm)	DEPT δ (in ppm)	Remark
1.	38.70	38.70	CH_2
2.	27.04	27.05	CH_2
3.	78.98	78.99	CH
4.	38.87	-	C (Quaternary Carbon)
5.	55.28	55.29	CH
6.	18.30	18.31	CH_2
7.	34.22	34.23	CH_2
8.	40.91	-	C (Quaternary Carbon)

Table 6: *(Continued)*

9.	50.39	50.40	CH
10.	37.16	-	C (Quaternary Carbon)
11.	20.83	20.84	CH_2
12.	25.20	25.21	CH_2
13.	37.30	37.31	CH
14.	42.71	-	C (Quaternary Carbon)
15.	27.39	27.40	CH_2
16.	33.97	33.98	CH_2
17.	-	-	-
18.	48.75	48.76	CH
19.	48.07	48.08	CH
20.	150.48	-	C (Quaternary Carbon)
21.	29.74	29.75	CH_2
22.	-	-	-
23.	27.99	28.00	CH_3
24.	16.12	16.13	CH_3
25.	15.98	15.99	CH_3
26.	15.37	15.38	CH_3
27.	14.76	14.77	CH_3
29.	109.71	109.71	CH_2
30.	19.08	19.08	CH_3
a.	20.65	20.65	CH_3
b.	29.18	29.18	CH_2
c.	47.79	47.79	
d.	52.61	52.61	
e.	53.84	53.84	
f.	59.39	-	C (Quaternary carbon)
g.	60.56	60.56	CH_2
h.	62.47	62.47	CH_2

Table 6: *(Continued)*

i.	69.42	69.42	
j.	70.38	70.38	
k.	128.58	128.58	CH
l.	129.52	129.52	CH
m.	133.57	133.57	CH

Below there is a table (**table 7**), that showed the comparison of **CM-2** with that of Lupeol (from the literature) and C**M-1**. Based on this information, lupeol has 30 carbons, **CM-1** has 31 carbons and **CM-2** has about maximum of 40 carbons. Even though the three compounds have different number of carbons, out of 40 carbons compound **CM-2** has about 27 carbons whose chemical shifts are closely resembles to that of lupeol and **CM-1**. The three compounds are in a good agreement with these 27 carbons which are used to propose the partial structure for **CM-2**. Therefore, based on this close similarity between the three compounds the partial structure of **CM-2** was proposed. In addition to this there is also close similarity in the chemical shifts of protons of **CM-2** with that of **CM-1**.This was also helped to propose the partial structure.

Table 7: Comparison of ^{13}C NMR (400 MHz, $CDCl_3$) spectral data of compound **CM-2** with Lupeol (from the literature [30, 31]) and **CM-1**.

Carbon No.	**CM-2**	**Lupeol**	**CM-1**
1.	38.70	38.70	38.72
2.	27.04	27.36	27.42
3.	78.98	78.65	79.00
4.	38.87	38.85	38.87
5.	55.28	55.26	55.31
6.	18.30	18.39	18.33
7.	34.22	34.26	34.29
8.	40.91	40.80	40.84
9.	50.39	50.34	50.45
10.	37.16	37.11	37.18

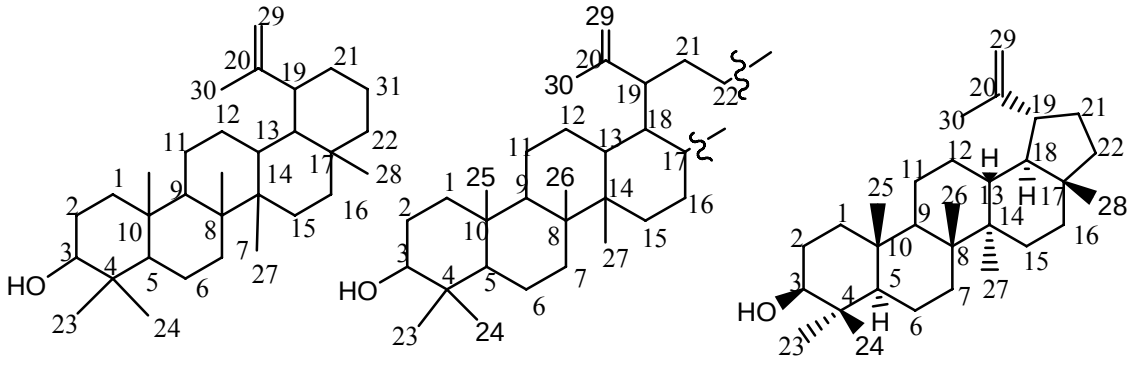
Table 7: *(Continued)*

11.	20.83	20.97	20.94
12.	25.20	26.53	25.15
13.	37.30	37.96	38.08
14.	42.71	42.97	43.01
15.	27.39	27.36	27.46
16.	33.97	35.44	35.59
17	-	42.74	42.84
18.	48.75	48.72	48.32
19.	48.07	43.77	48.00
20.	150.48	151.32	150.97
21.	29.74	31.70	29.86
22.	-	39.83	40.01
23.	27.99	28.03	27.99
24.	16.12	16.11	16.12
25.	15.98	15.96	15.98
26.	15.37	15.48	15.37
27.	14.76	14.52	14.56
28.	-	17.69	18.01
29.	109.71	109.67	109.33
30.	19.08	19.65	19.31
31.	-	-	29.71
a.	20.65	-	-
b.	29.18	-	-
c.	47.79	-	-
d.	52.61	-	-
e.	53.84	-	-
f.	59.39	-	-
g.	60.56	-	-
h.	62.47	-	-

Table 7: *(Continued)*

i.	69.42	-	-
j.	70.38	-	-
k.	128.58	-	-
l.	129.52	-	-
m.	133.57	-	-

Based on the **table 7** above, the 17th and 22nd positions were not assigned with the chemical shifts of carbons in the partial structure of compound **CM-2**. This was because of those unknown side chains which were attached to C-17 and C-22. The position of C-21 was assigned based on the information which was obtained from 2D spectral data mainly ^{1}H-^{1}H COSY which showed the correlation of H-19 with that of H-21. The 17th position in lupeol from the literature and **CM-1** was quaternary carbon but in the partial structure of compound **CM-2** is not quaternary. An evidence for this was obtained from 2D spectral data. Therefore, based on this information the 17th position in the compound **CM-2** is not quaternary.



CM-1 **CM-2** **Lupeol**

In addition to the above informations which were used to compute the chemical shifts of the partial carbons of **CM-2** with that of lupeol from the literature and compound **CM-1**,

there are also 2D experiments which were used to support the above predictions for given structure of compound **CM-2**.

The ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum (Appendix 2.6) showed strong correlations between H-3 with that of H-2, H-30 with that of H-29, H-19 with that of H-21, H-5 with that of H-6. Similarly, there are also other correlations which were observed from COSY spectrum as shown below in **table 8**.

Table 8: ^{1}H NMR and some selected ^{1}H-^{1}H COSY (400 MHz, $CDCl_3$) spectral data of compound **CM-2** (Partially)

Carbon No	^{1}H NMR δ (in ppm)	^{1}H-^{1}H COSY
1.	1.60 and 0.90	
2.	1.62	$H^2 \leftrightarrow H^3$
3.	3.20	$H^3 \leftrightarrow H^2$
5.	0.70	$H^5 \leftrightarrow H^6$
6.	1.58	$H^6 \leftrightarrow H^5$
7.	1.50	
9.	1.30	
11.	1.20	$H^{11} \leftrightarrow H^{12}$
12.	1.65	$H^{12} \leftrightarrow H^{11}$
13.	1.64	
15.	1.60	
16.	1.90	
18.	1.70	
19.	2.40	$H^{19} \leftrightarrow H^{21}$
21.	1.92 and 1.40	$H^{21} \leftrightarrow H^{19}$
23.	1.00	
24.	0.78	
25.	0.85	
26.	1.05	

Table 8: *(Continued)*

27.	1.00	
28.	4.60 and 4.70	$H^{29} \leftrightarrow H^{30}$
29.	1.78	$H^{30} \leftrightarrow H^{29}$

Heteronuclar Single Quantum Correlation (HSQC) spectrum (Appendix 2.7) also correlates the chemical shift of proton with directly bonded carbon atoms. This experiment showed the correlations in the partial structure of compound **CM-2** such that, the two diastereotopic methylene protons at δ 4.6 and δ 4.7 correlate with that of carbon at δ 109.71. This correlation showed that this carbon is a terminal olefinic carbon. Other diastereotopic methylene protons at δ1.40 and δ1.92 correlate to that of carbon at δ 29.74. Other diastereotopic methylene protons at δ 1.60 and δ 0.90 also correlate with that of carbon at δ 38.70. In the same manner, the HSQC spectrum also showed different kinds of correlations of the protons with that of carbon as shown in Appendix 2.7.

Another experiment which was used to propose the partial structure of the compound **CM-2** is HMBC experiment (Appendix 2.8). From this HMBC spectrum, there are correlations of protons with carbons which are two or three bonds away. Some of the selected correlations which were observed from HMBC spectrum can be shown in the table below.

Table 9: Observed Correlations in HMBC (400 MHz, $CDCl_3$) spectral data of compound **CM-2** (Partially)

Carbon No.	C NMR δ (in ppm)	HMBC
1.	38.70	
2.	27.04	
3.	78.98	
4.	38.87	
5.	55.29	
6.	18.30	
7.	34.22	

Table 9: *(Continued)*

8.	40.91	
9.	50.39	
10.	37.16	
11.	20.83	
12.	25.20	
13.	37.30	
14.	42.71	
15.	27.39	
16.	33.97	
17.	-	
18.	48.75	
19.	48.07	
20.	150.94	
21.	29.74	
22.	-	
23.	27.99	$H^{23}\leftrightarrow$ C-3, C-5, C-4, C-2, C-24, C-1
24.	16.12	$H^{24}\leftrightarrow$C-3, C-5, C-4, C-23
25.	15.98	$H^{25}\leftrightarrow$ C-5, C-9, C-1
26.	15.37	$H^{26}\leftrightarrow$ C-9, C-14, C-8, C-15
27.	14.76	$H^{27}\leftrightarrow$C-8, C-14
29.	109.71	$H^{29}\leftrightarrow$ C-19, C-30
30.	19.08	$H^{30}\leftrightarrow$ C-20, C-29, C-19, C-18

As shown in the **table 9** above, no more correlations were observed from HMBC spectrum. This limitation causes the challenges to propose the full structure for **CM-2**. Therefore, based on those few correlations which were observed from the HMBC spectrum as shown in the table above, only the partial structure of **CM-2** was proposed.

Hence, based on the HMBC spectrum of **CM-2**, the correlations of the protons with that of carbons can be shown structurally as follows:

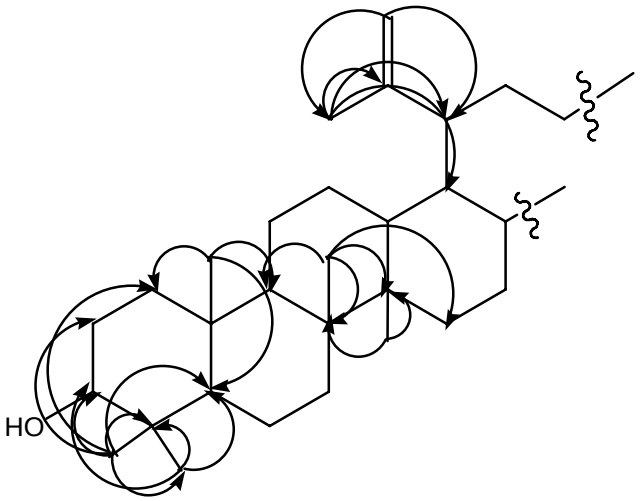
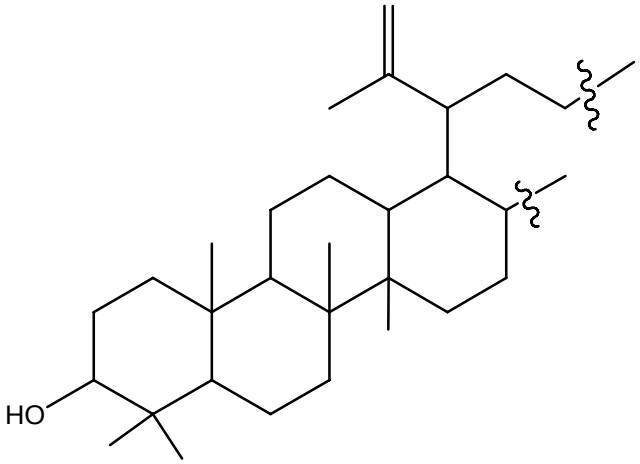


Figure 2: Observed HMBC correlations of Partial structure of compound **CM-2**

Based on the spectroscopic data and the literature, the compound **CM-2** is a terpenoid with the following partial structure.



Partial structure of compound CM-2

6. EXPERIMENTAL

6.1. General

^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker Advance 400 MHz spectrometer in $CDCl_3$ with TMS as internal started. The ultra-violet and visible (UV-Vis) spectra were taken on GENESY'S 2PC UV-Vis scanning spectrometer (200-800 nm). Infrared (IR) spectra were obtained on Perkin-Elmer BX infrared spectrometer (400-4000 cm^{-1}) using KBr. Melting points were recorded using Thomas HOOVER capillary melting point apparatus. Analytical thin layer chromatograms were run on a ready made 0.2 mm thick layer of Merck silica gel 60 F_{254} coated on aluminum foil. Compounds on TLC were detected after spraying 1% vanillin sulphuric acid and after heating for a few minutes.

6.2. Plant Material

The stem barks of the *Croton macrostachyus* were collected from Amhara Regional State, Awi Administrative Zone (Gojjam), Ankesha Guagusa Woreda, particularly from Azena, which is 450 km from Addis Ababa on December 2006. Voucher specimen is deposited in the National Herbarium, Ethiopia, Department of Biology, Addis Ababa University (Voucher no. : ZYD 001)

6.3. Extraction and Isolation

The air dried and powdered plant material (145g) was first soaked with 450ml petroleum ether for 72 hours and the extract was collected and discarded. The solvent free marc was then soaked with 400ml of dichloromethane for 36 hours and the extract was collected. This filtrate was evaporated under reduced pressure using the Rotavapor and afforded 2g pale yellow solid residue which on TLC solvent system chloroform: ethyl acetate (9:1) showed two coloured spots.

This 2g crude extract was dissolved in chloroform and applied to a silica gel (60g) column chromatography which was packed with chloroform (100%). The column was eluted using the following solvent systems; chloroform (100%): fractions 1-5,

chloroform: ethyl acetate (9:1): fractions 6-13, chloroform: ethyl acetate (4:1): fractions 14-20, chloroform: ethyl acetate (7:3): fractions 21-24, chloroform: ethyl acetate (1:1): fractions 25-26, and ethyl acetate (100%): fraction 27. Totally 27 fractions were collected each 50ml.

Out of 27 fractions which were collected using the solvent systems increased polarity, only those fractions from 10- 19 showed the characteristic coloured spots on TLC upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. The remaining fractions (1-9 and 20-27) did not show the characteristic coloured spots on TLC upon spraying 1% vanillin sulphuric acid and after heating for a few minutes.

Among fractions 10-19, fraction 12 showed single spot on TLC using the solvent system chloroform: ethyl acetate (4:1) upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. This fraction was pure and after the removal of the solvent using the Rotavapor afforded 56mg of the compound **CM-1**.

In addition fraction 16 also showed characteristic coloured spots on TLC using the solvent system chloroform: ethyl acetate (4:1) and upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. However, the TLC examination showed two spots. Based on this TLC examination this fraction was not pure and it was purified by soaking with petroleum ether. The extract was collected by decantation and the remaining residue, which was not soluble in petroleum ether, afforded 23mg compound **CM-2** after drying using the Rotavapor.

6.4. Spectral Data

Compound CM-1: white solid, m.pt. 181-187 ^{0}C. IR ν_{max} (KBr) cm^{-1}**,** 3308, 3066, 2946, 2873, 2851, 1638, 1453, 1380, 879, 1043. UV-Vis λ_{max} ($CHCl_3$) 268 nm. ^{1}H NMR (400MHz, $CDCl_3$): 4.6 (1H, s, H-29) and 4.7 (1H, s, H-29), 3.2 (1H, dd, H-3), 2.4 (1H, m, H-19), 2.15 (1H,s, OH), 1.90 (1H, m, H-21), 1.75 (3H, brs, H-30), 1.03 (3H, s, H-26), 0.84 (3H, s, H-25), 0.97(3H, s, H-27), 0.95 (3H,s, H-23), 0.79 (3H, s, H-28), 0.76 (3H, s, H-24), 0.7 (1H, d, H-5). ^{13}C NMR (400 MHz, $CDCl_3$), 38.72 (C-1), 27.42 (C-2), 79.00 (C-3), 38.87 (C-4), 55.31 (C-5), 18.33 (C-6), 34.29 (C-7), 40.84 (C-8), 50.45 (C-9), 37.18 (C-10), 20.94 (C-11), 25.15 (C-12), 38.07 (C13), 43.01 (C-14), 27.46 (C-15), 35.59 (C-16), 42.84 (C-17), 48.32 (C-18), 48.00 (C-19), 150.97 (C-20), 29.86 (C-21), 40.01 (C-22), 28.00 (C-23), 16.12 (C-24), 15.98 (C-25), 15.37 (C-26), 14.56 (C-27), 18.01 (C-28), 109.33 (C-29), 19.31 (C-30), 29.71 (C-31).

Compound CM-2 (Partial): White solid, m.pt 194-197 ^{0}C. IR ν_{max} (KBr) cm^{-1}, 3377, 2940, 2868, 1463, 1452, 1376, 1283, 1237, 1214, 1120, 1043, 1026, 721. UV-Vis λ_{max} ($CHCl_3$) 271 nm. ^{1}H NMR (400 MHz, $CDCl_3$); 4.6 (1H, s, H-29) and 4.7 (2H, s, H-29), 3.2 (1H. dd, H-3), 2.4 (1H, m, H-19), 0.70 (1H, d, H-5), 1.78 (3H, brs, H-29), 1.00 (6H, s, H-23 and H-27), 0.78 (3H, s, H-24), 0.85 (3H, s, H-25), 1.05 (3H, s, H-26), 0.90 and 1.60 (2H, complex, H-1). ^{13}C NMR (400 MHz, CDCl3); 38.70 (C-1), 27.04 (C-2), δ78.98 (C-3), 38.87 (C-4), 55.28 (C-5), 18.30 (C-6), 34.22 (C-7), 40.91 (C-8), 50.39 (C-9), 37.16 (C-10), δ20.83 (C-11), 25.20 (C-12), 37.30 (C-13), 42.79 (C-14), 27.39 (C-15), 33.97 (C-16), 48.75 (C-18), 48.07 (C-19), 150.48 (C-20), 29.74 (C-21), 27.99 (C-23), 16.12 (C-24), 15.98 (C-25), 15.37 (C-26), 14.76 (C-27), 109.71 (C-29), 19.08 (C-30).

7. CONCLUSION

Two terpenoids were isolated and characterized from the stem bark of the *Croton macrostachyus*. The two compounds were isolated for the first time from this plant. Compound **CM-2** has carbon atoms much greater than 30 and only the partial structure was reported because of the limitations of the HMBC experiment which showed only the limited correlations of protons with that of carbons two or three bonds away.

The intensive coupling, overlapping of signals and coincidence of magnetically non equivalent protons made a difficulty to differentiate one from another during correlation of different experimental systems in both compounds.

In general, the compounds were not UV sensitive using different solvent systems. They only showed spots on TLC upon spraying 1% vanillin sulfuric acid and after heating for a few minutes.

8. REFERENCES

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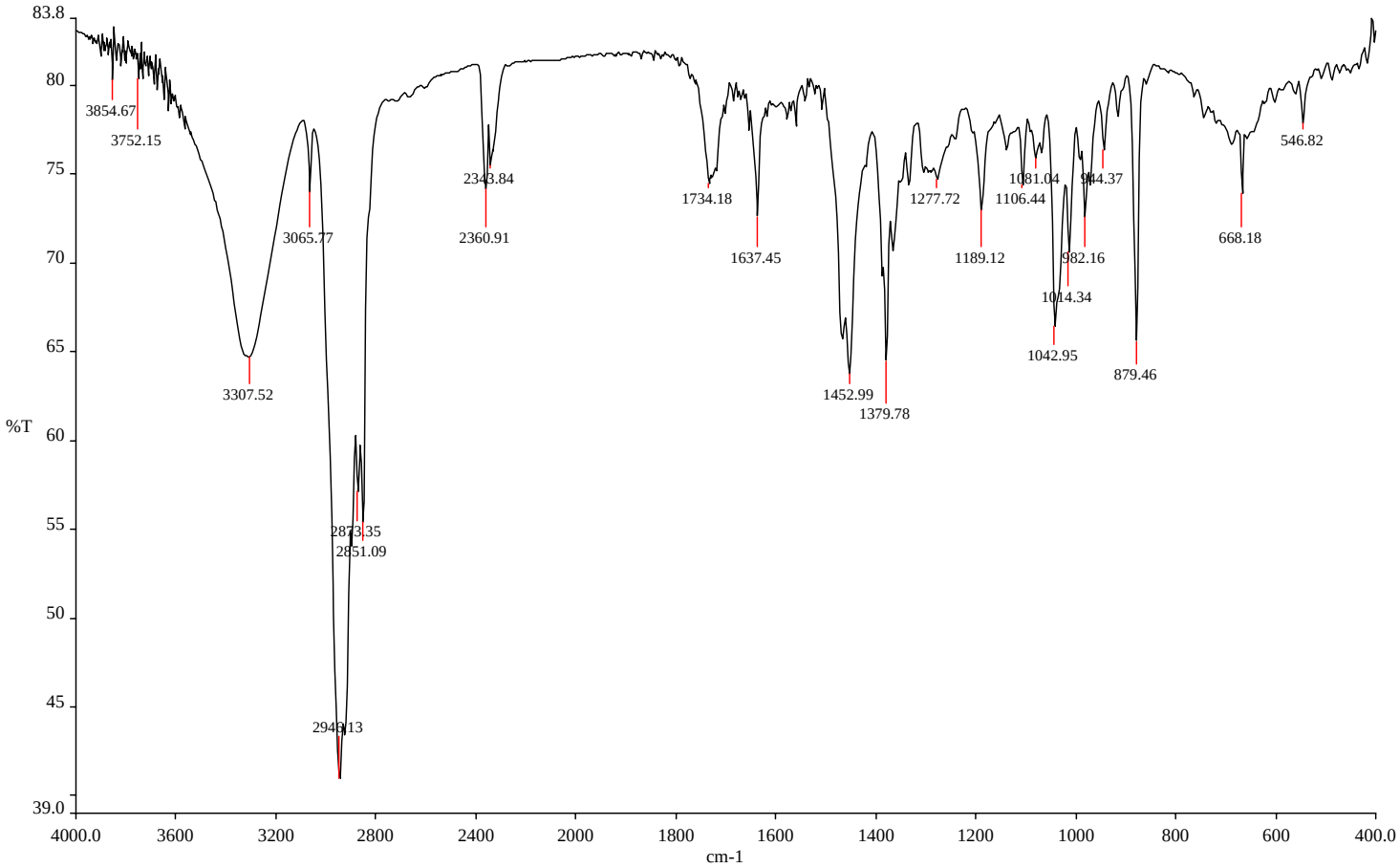
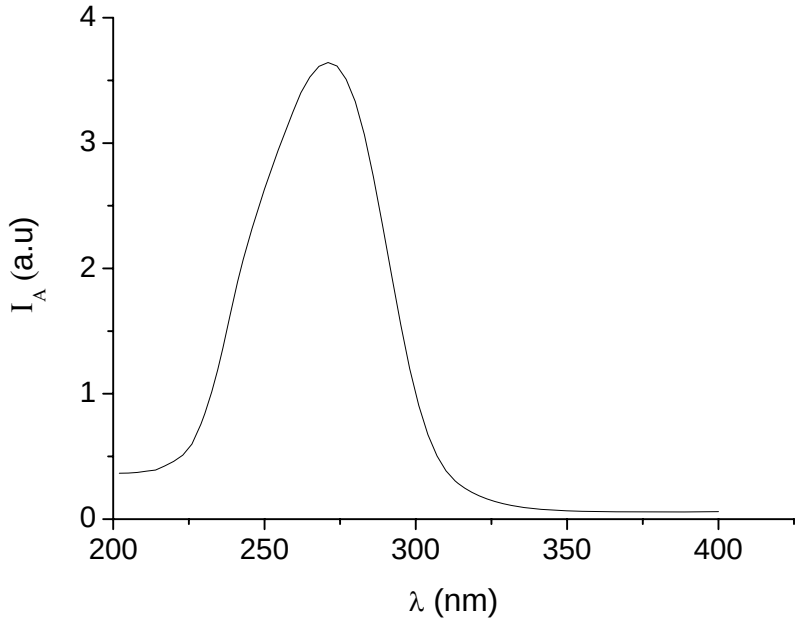
APPENDIX

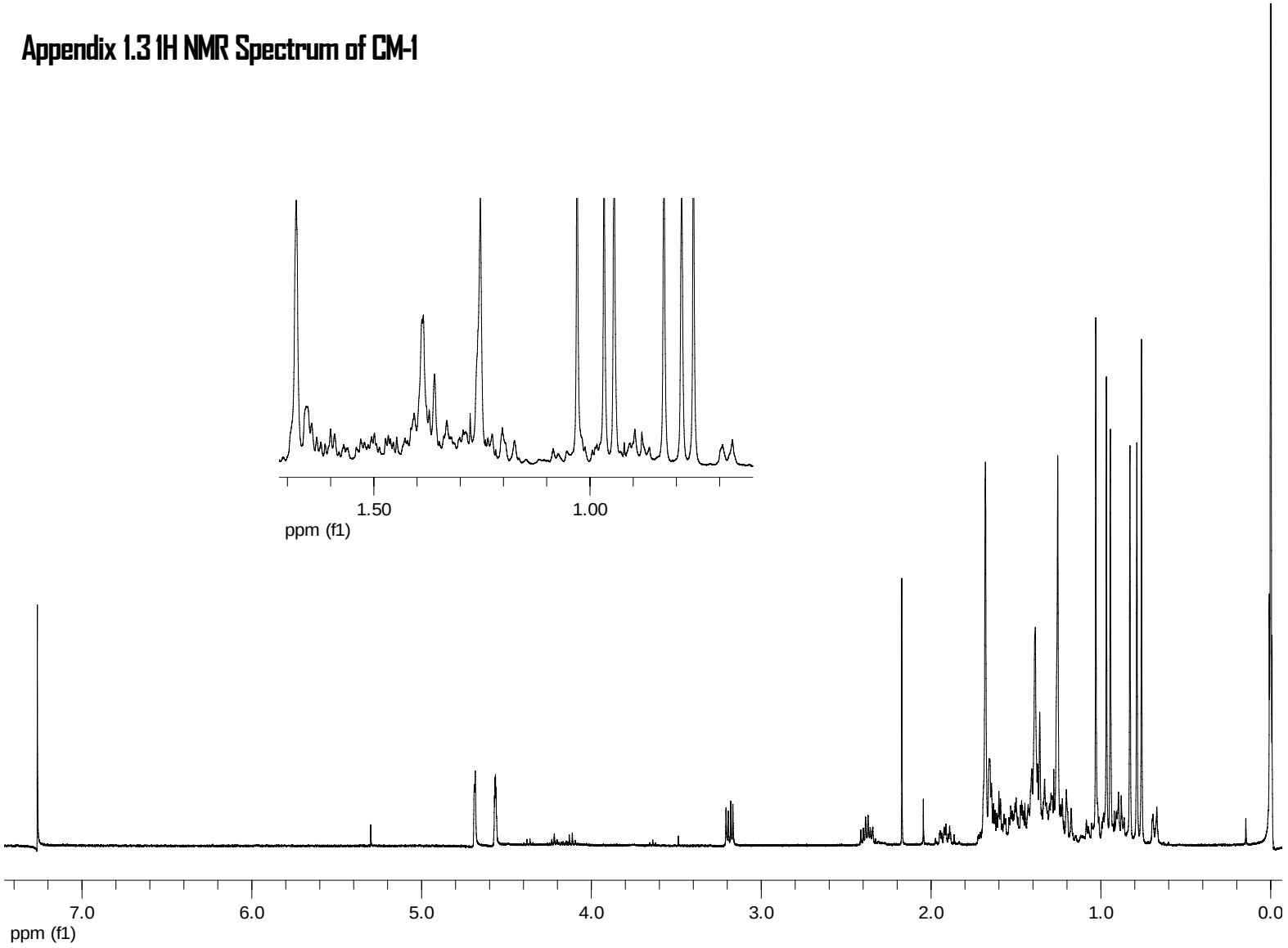
Appendix 1.1 IR spectrum of CM-1



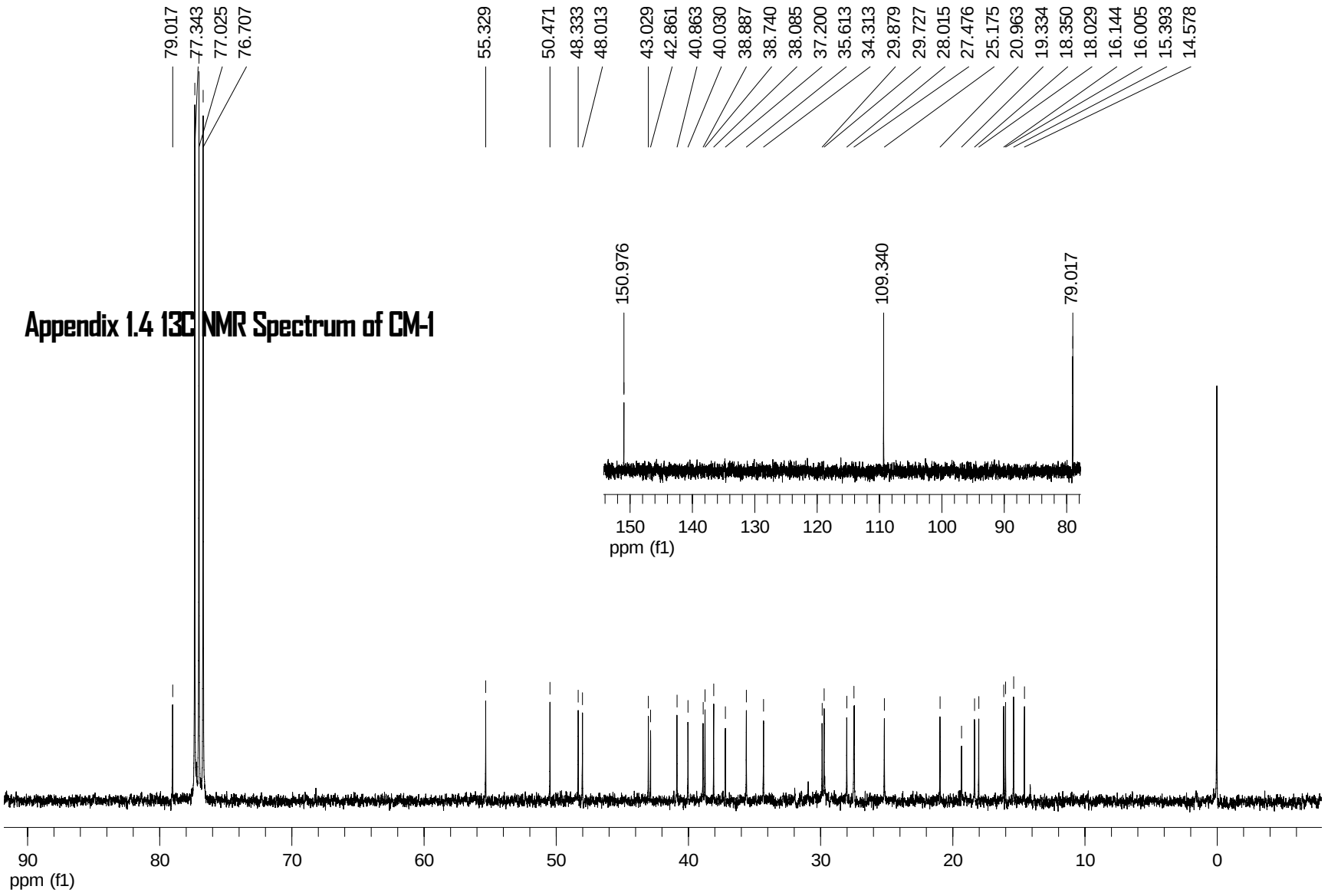
Appendix 1.2 UV Spectrum of CM-1



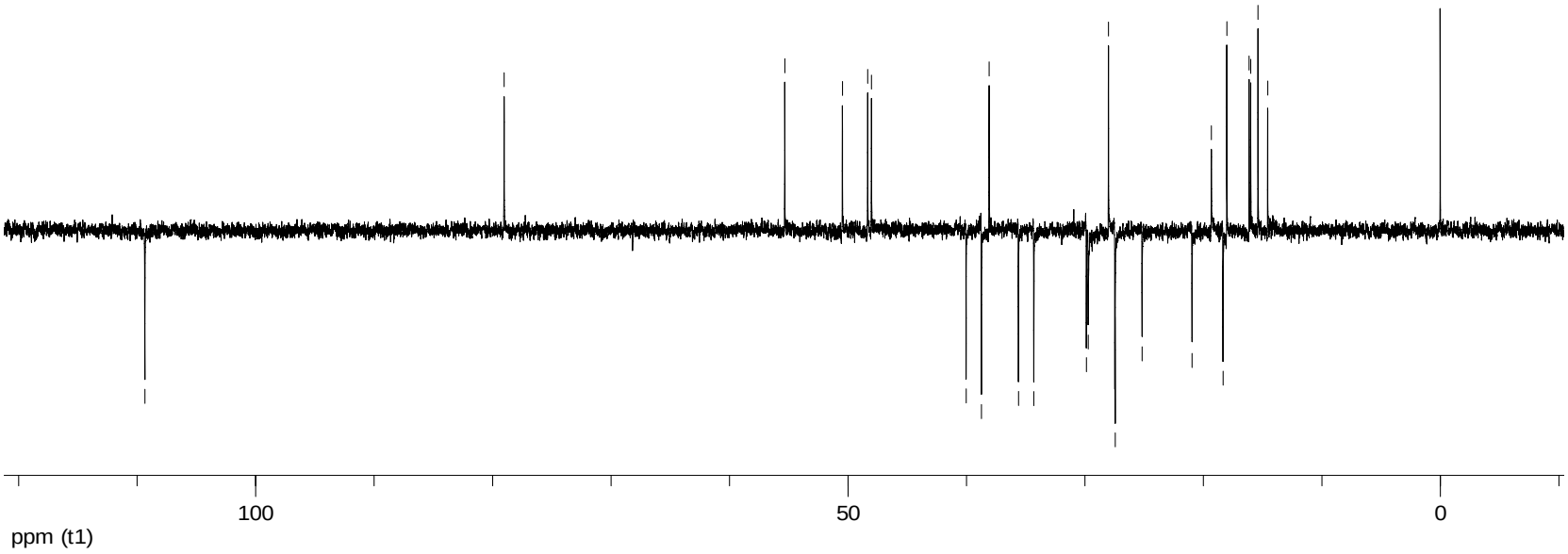
Appendix 1.3 1H NMR Spectrum of CM-1

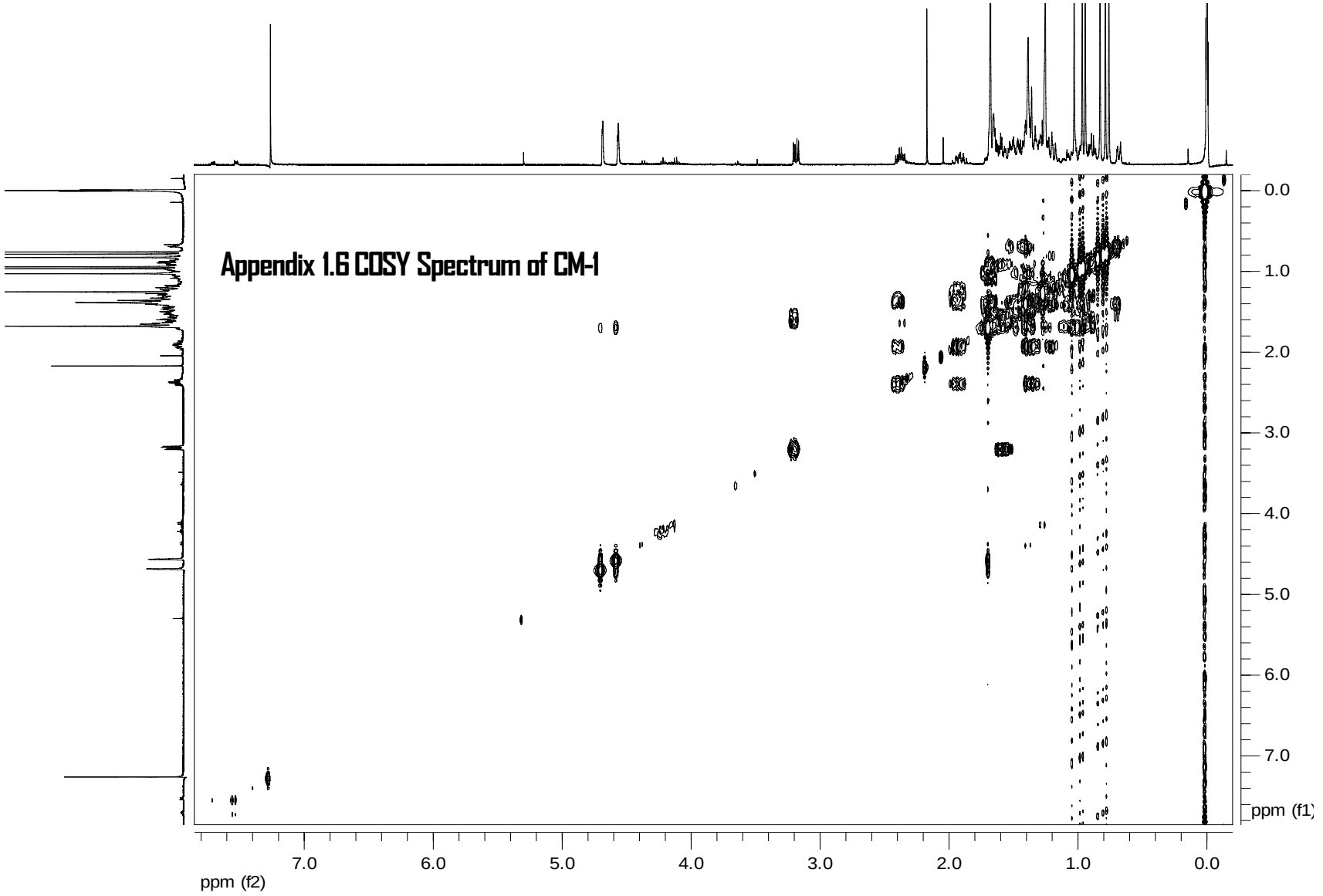


Appendix 1.4 13C NMR Spectrum of CM-1

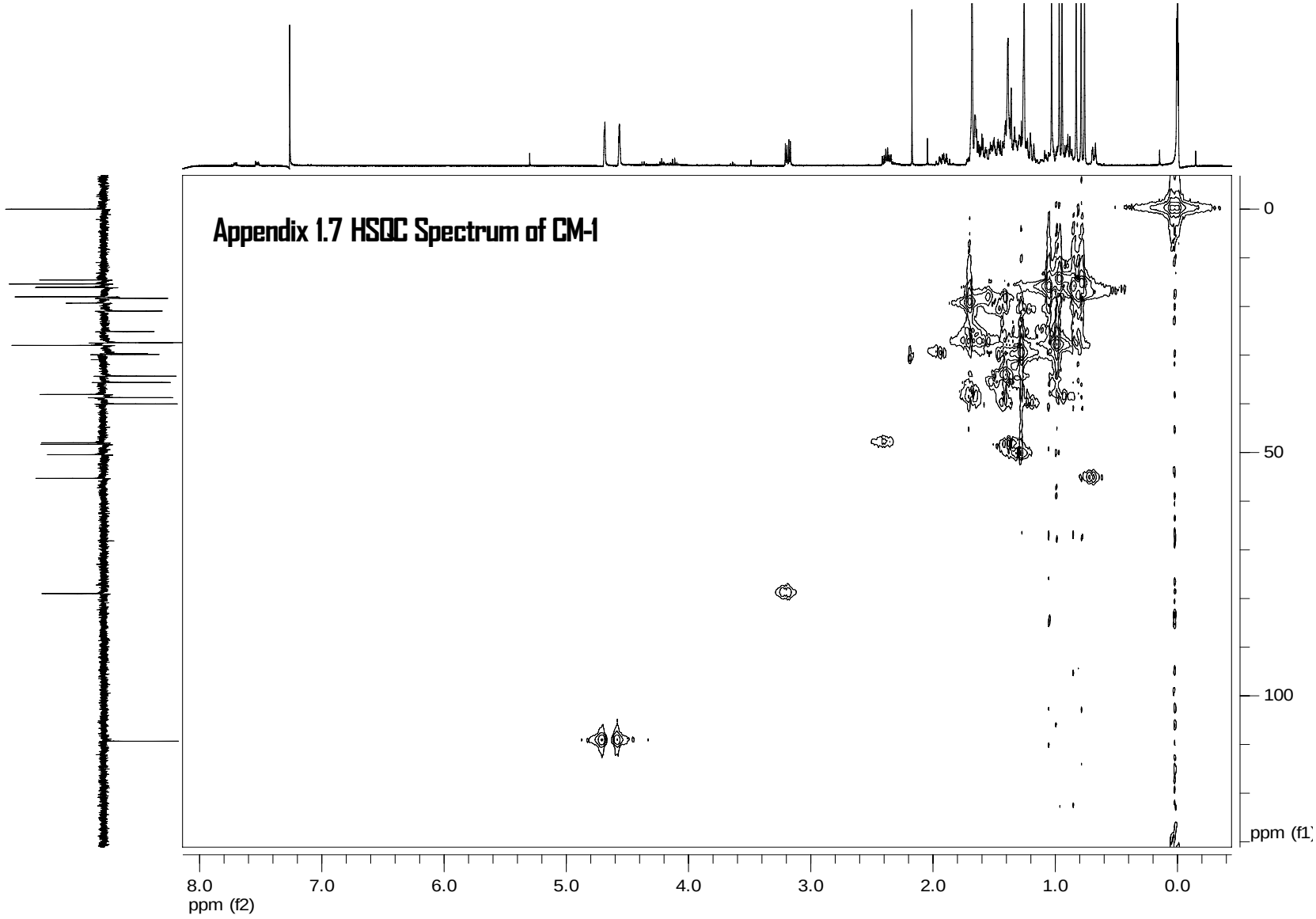


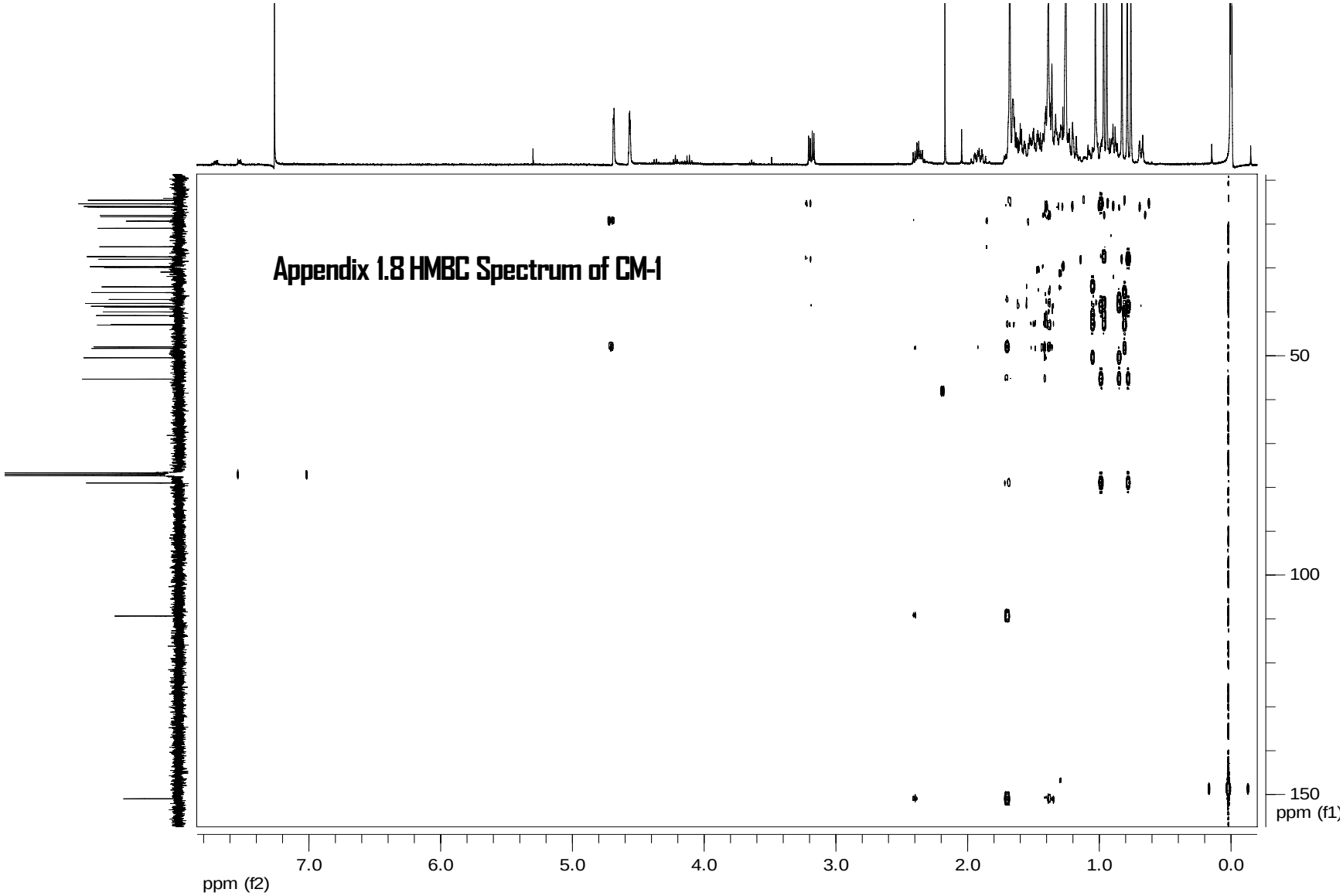
Appendix 1.5 13C NMR (DEPT) Spectrum of CM-1



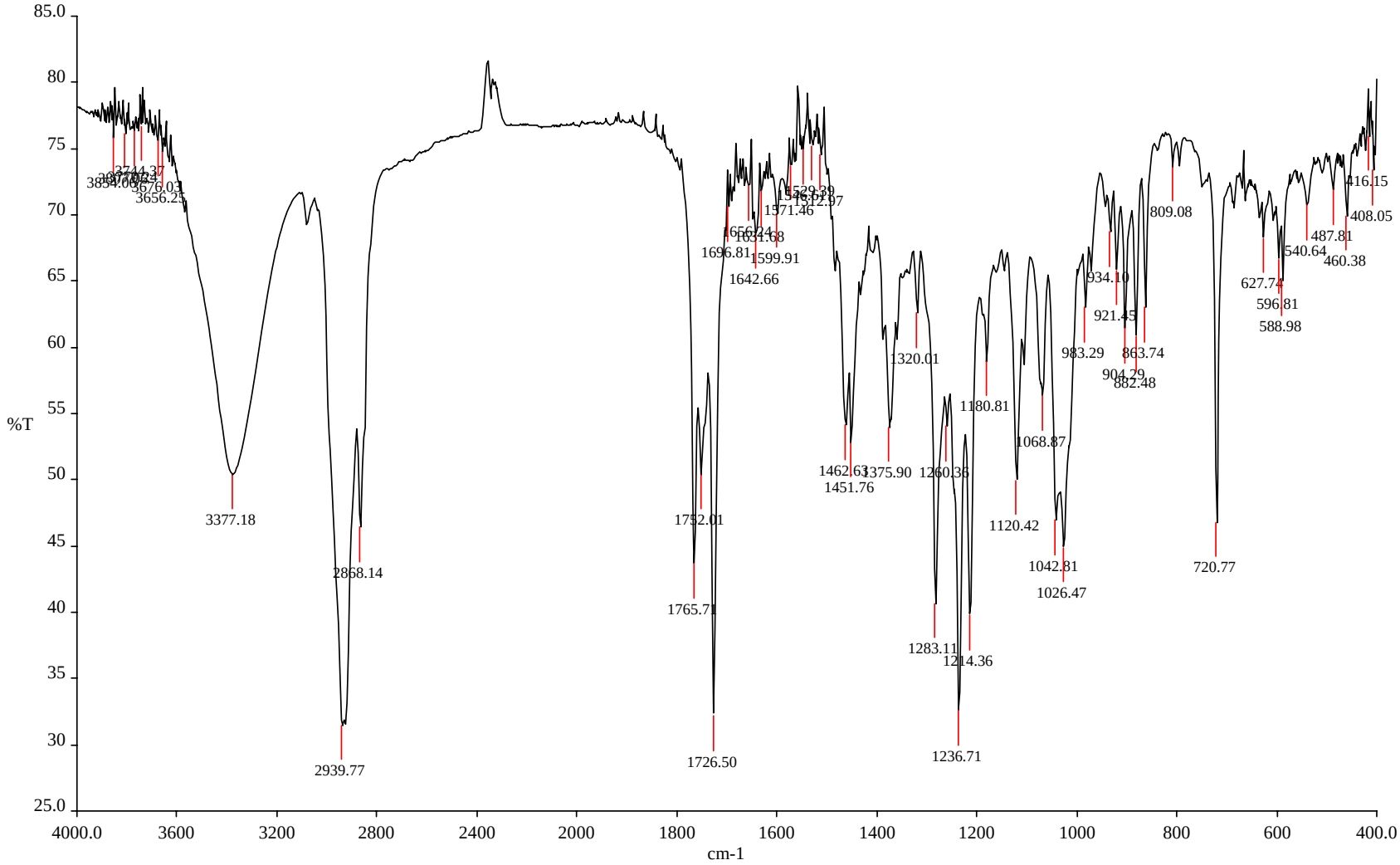

Appendix 1.6 COSY Spectrum of CM-1
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f2)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
ppm (f1)

Appendix 1.7 HSQC Spectrum of CM-1

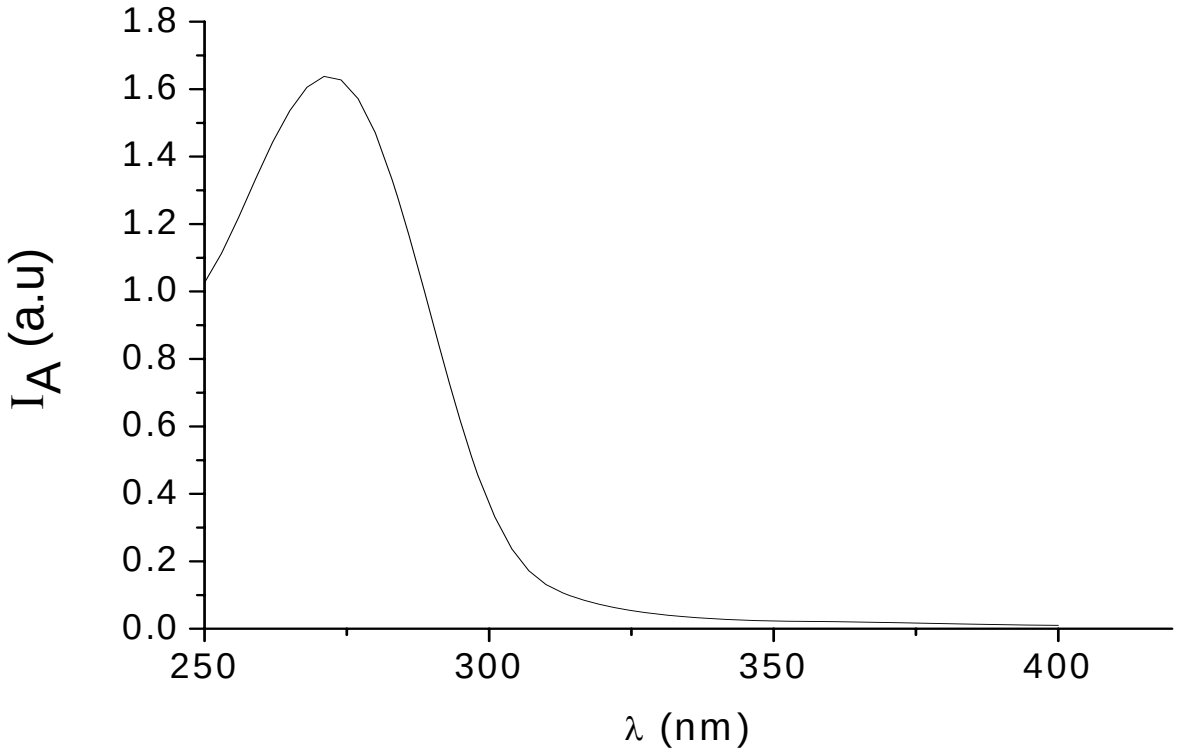



Appendix 1.8 HMBC Spectrum of CM-1
50
100
150
ppm (f1)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f2)

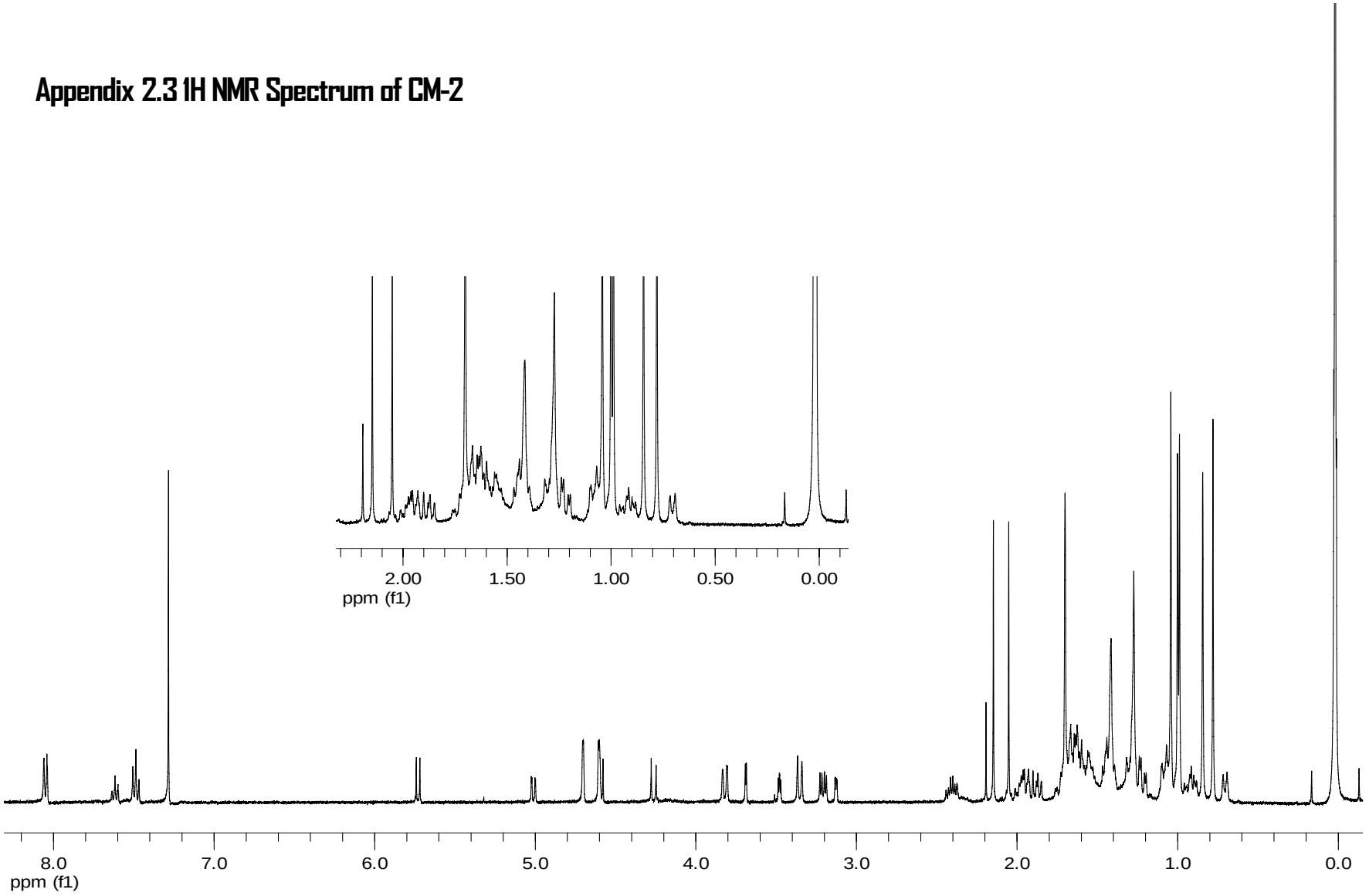
Appendix 2.1 IR Spectrum of CM-2



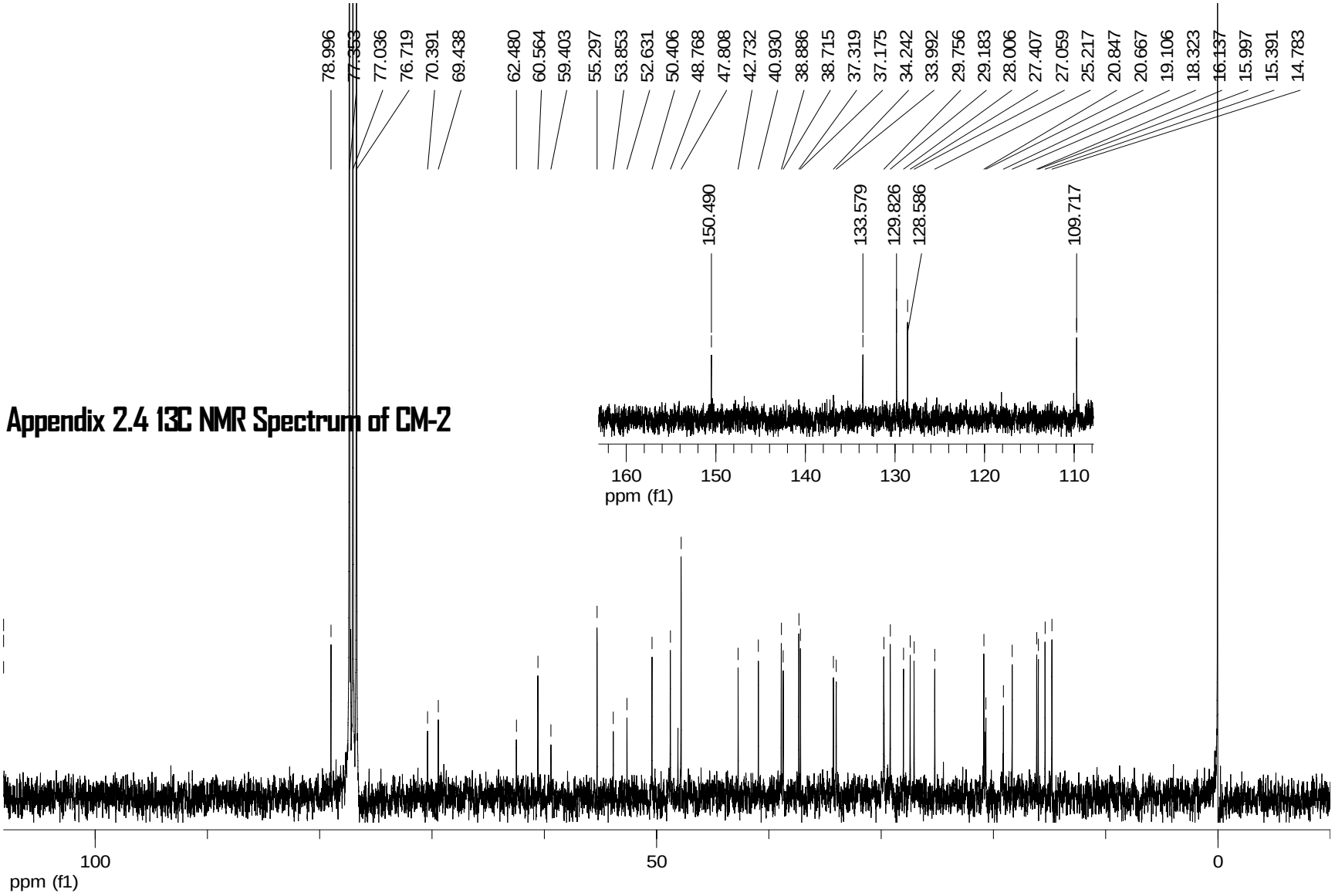
Appendix 2.2 UV Spectrum of CM-2

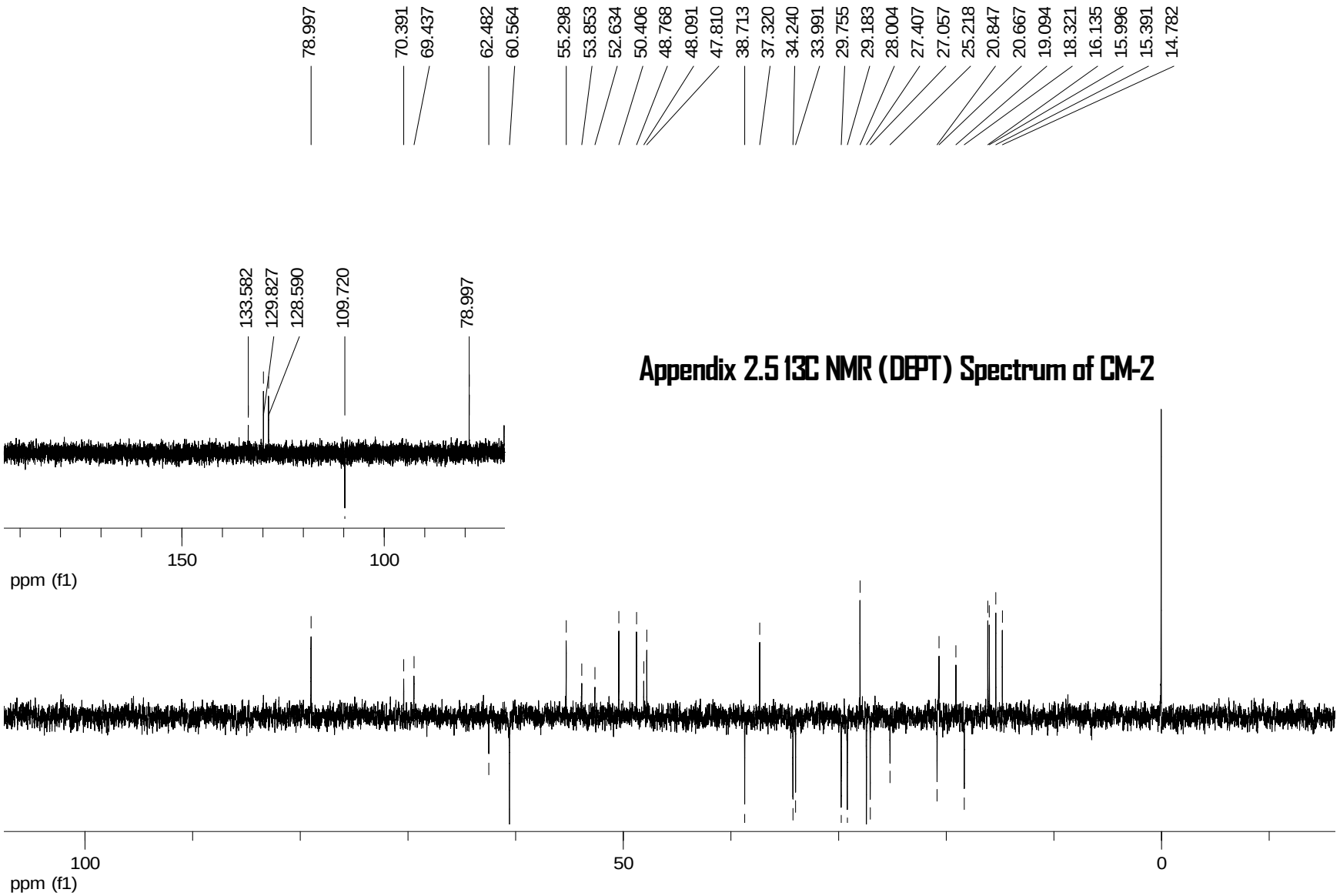


Appendix 2.3 1H NMR Spectrum of CM-2

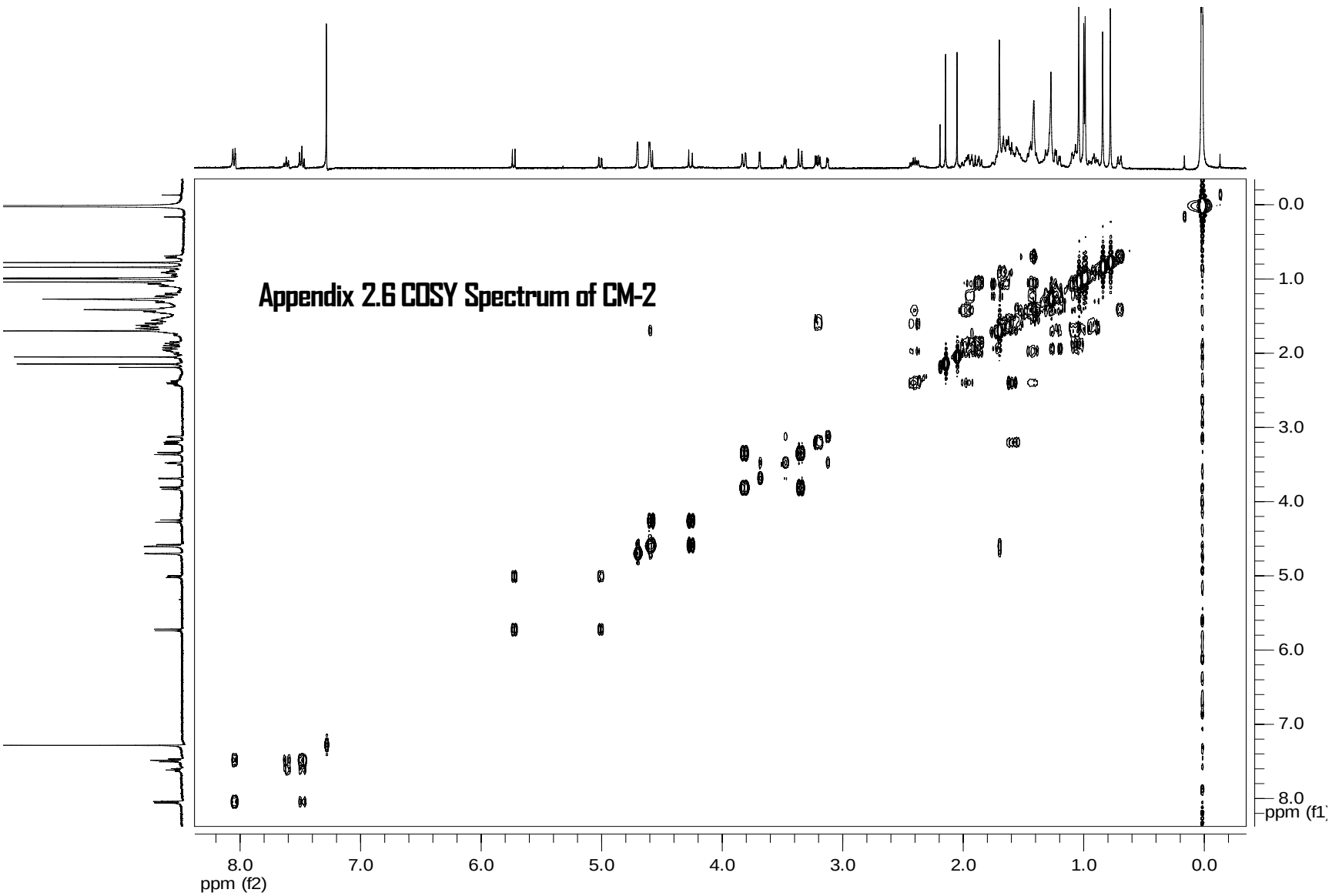


Appendix 2.4 13C NMR Spectrum of CM-2

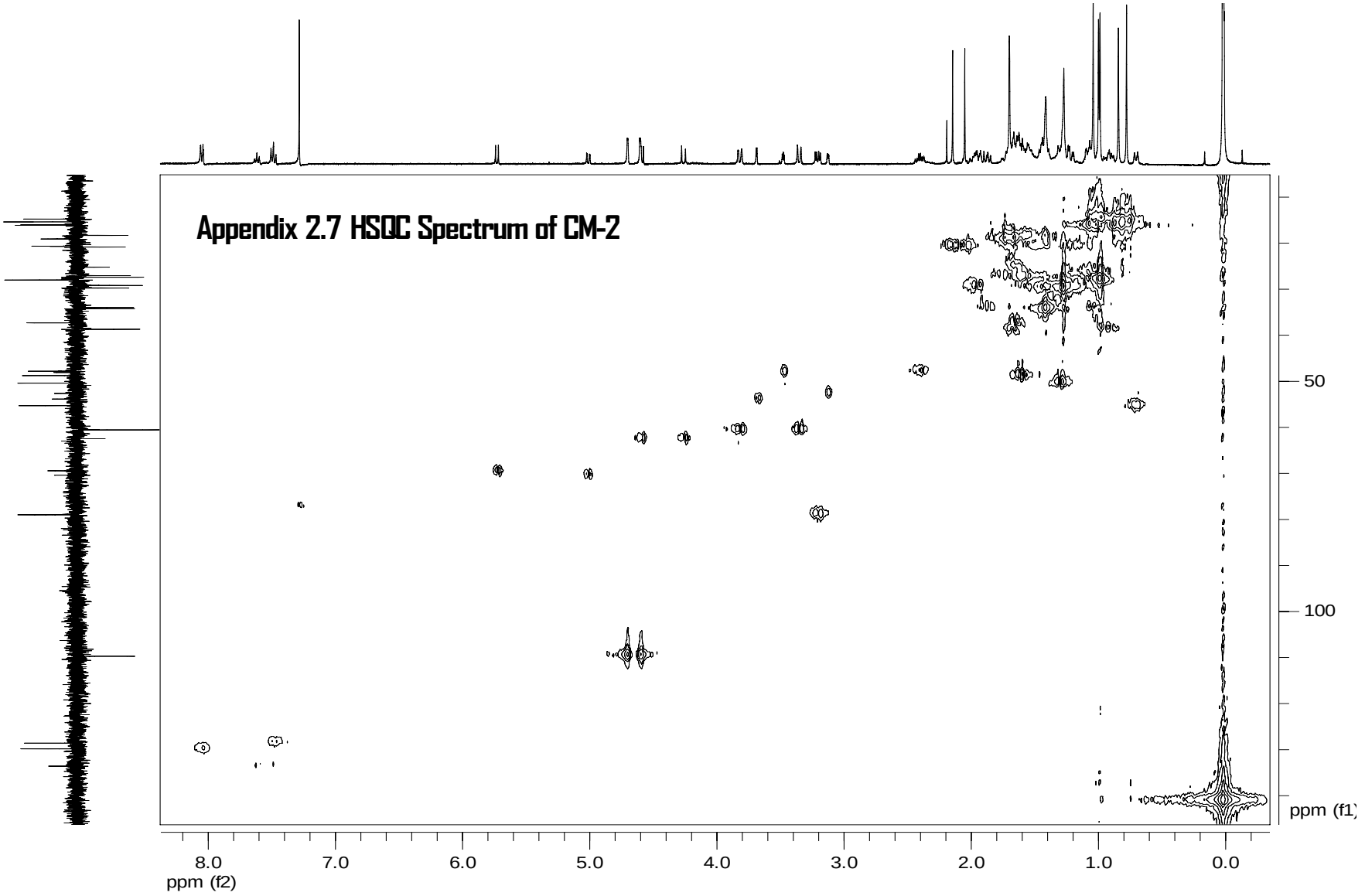


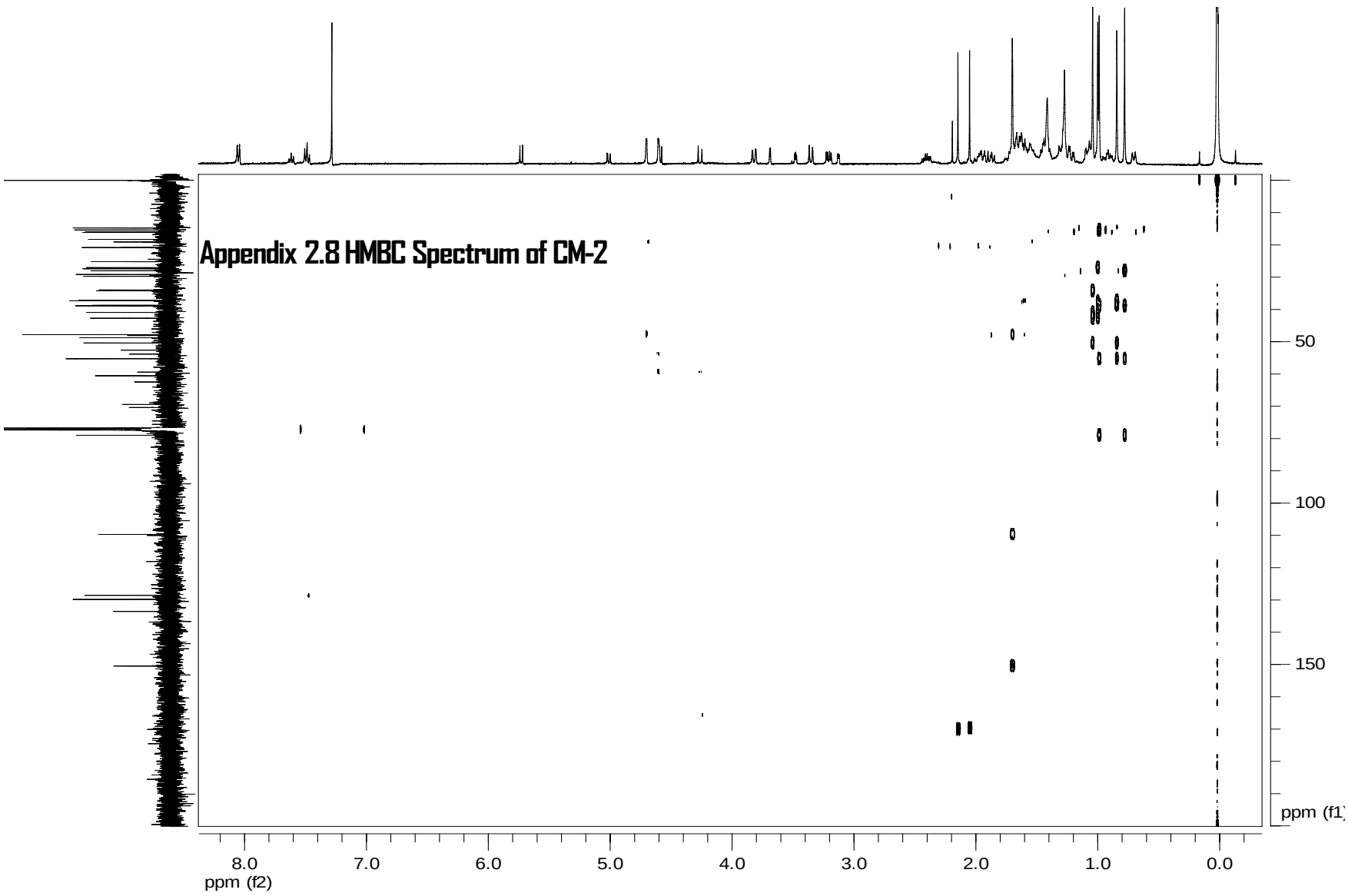


Appendix 2.5 13C NMR (DEPT) Spectrum of CM-2


Appendix 2.6 COSY Spectrum of CM-2
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f2)
ppm (f1)

Appendix 2.7 HSQC Spectrum of CM-2




Appendix 2.8 HMBC Spectrum of CM-2
50
100
150
ppm (f1)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f2)